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The Japanese beetle and neem : efficacy of commercial formulations on laboratory and field populations.

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THE JAPANESE BEETLE AND NEEM: EFFICACY OF COMMERCIAL FORMULATIONS ON LABORATORY AND FIELD POPULATIONS

A Thesis Presented

by

SUSAN J. ROY

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DEDICATION

This Thesis is dedicated to:

Bobbie Roy

nee: Anne-Marie Robertine Pelletier

my mother

whose delight in all that I did as a child

and acceptance of the many hours I spent in the woods

as well as of all I brought home from there

made all the difference

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CHAPTER I

LITERATURE REVIEW

Introduction

The use of natural plant extracts to kill or repel insect pests, utilizing the allelochemicals contained in these extracts, has a history as old as agriculture (Pennington 1975). Of these phytochemical pesticides, one of the most extensively studied in recent years has been azadirachtin, which is derived from the Indian neem tree, *Azadirachta indica* Jussieu. This plant has been used in India and Burma for centuries to control pests (Koul et al. 1990). Articles detailing some of the insecticidal qualities of extracts of this tree were published as early as the 1920's by Indian scientists (Anon.1992), but the first western scientist to write about neem tree extracts was Heinrich Schmutterer of the Institute of Phytopathology and Applied Zoology in Giessen, West Germany. During a 1959 trip with his students to the Sudan to study the desert locust, *Schistocerca gregaria* Forskal, Schmutterer noticed that locusts swarming through an area were leaving neem trees untouched while consuming most other foliage in the area. He observed that the locusts would settle and taste the foliage, then fly on to other food sources (Anon. 1992).

Extracts of the leaves, bark and seeds of the neem tree have been tested extensively since then for insecticidal impact on more than 200 species of plant pests (Anon. 1992). Related species have also been investigated, with the most common, *Melia azedarach* L., receiving a good deal of attention during the 1980's (Olkowski & Olkowski 1988). More recently, Schmutterer has begun investigating the insecticidal activity of the Philippine neem tree, *Azadirachta*

integrifolia Merritt, which grows in areas of high rainfall (Schmutterer 1989) and has much larger seeds than those of the Indian neem tree (van der Nat et al. 1991).

Because the primary active ingredients of the neem tree (azadirachtin and its isomers) have never been synthesized, all commercial formulations of neem are extracts of *A. indica*. Studies of *M. azaderach* have not been as extensive because of the presence of other constituents, including the meliatoxins, which are highly toxic to mammals (Ascher 1993). Ruscoe (1972) disputes the presence of azadirachtin in *M. azaderach*. Toxicological studies of the effects of extracts of *A. integrifolia* must be conducted before it can be widely utilized.

Extensive research on Indian neem tree extracts has been conducted in the last 30 years (Mordue 1993). Many changes in the style and approach of the research have occurred as the information about these extracts has accumulated. Reports of efficacy from studies conducted in the 1970's and early 1980's, especially against various leaf chewing insects, described treatments utilizing different parts of the neem tree extracted in several ways (Steets 1975). Scientists in many Third World countries approached the development of neem seed extracts with an emphasis on the needs of the farmers and cooperatives who were interested in the production of neem and its possible use as an alternative to commercially available pesticides. As a consequence, the emphasis of much of this early work was on the efficacy of crude extracts, and not on the development and comparison of standardized formulations (Schmutterer 1980).

Although work on the separation and identification of the many compounds found in neem began in India in 1942 (Koul et al. 1990), it was not until analytical methods were developed to identify and quantify the active

ingredients (Schmutterer et al. 1981, Schmutterer & Ascher 1984, 1987) that comparisons among these reports became possible. The information was limited by what was known about the amount of active ingredient, which was usually assumed to be azadirachtin and its isomers, in each extract.

Commercial formulations of neem seed kernel extract have subsequently been developed in India, Europe, and the United States. In the United States, the first Environmental Protection Agency registration for an azadirachtin-based pesticide was granted to Vikwood Botanicals Inc. of Sheboygan, WI (Larson 1989, 1990) for a formulation containing 0.3% azadirachtin and 14% neem oil. This product, known as Margosan-O™, is currently produced by W. R. Grace of Columbia, MD. The Ringer Corp. (Minneapolis, MN) also distributes Margosan-O under different trade names for the home market. Another American company, Agridyne Technologies of Salt Lake City, UT, has registered a similar product known as Azatin™ or Turplex™, depending on the product market and supplier. The availability of these products has changed the nature of research since the late 1980's, with better comparisons of efficacy now possible as a result of the standardization of the extract in these formulations (Jiliani & Saxena 1990, Stark 1992, Thomas et al. 1992). Other work involving the evaluation of neem seed oils (Isman et al. 1990, Jackai & Oyediran 1991) for azadirachtin content will expand the potential for other kinds of formulations for the commercial market.

Another difficulty in the evaluation of neem products prior to 1990 was the limited availability of pure azadirachtin. It was first offered as a 95% powder by Sigma Chemical Company in 1990. Although a simplified method of isolating azadirachtin had been published before then (Schroeder & Nakanishi 1987), most researchers had previously obtained purified azadirachtin from one of several large research groups with access to neem seeds, including those

headed by Rembold in Germany, Morgan in the United Kingdom, Koul in Canada, and Jacobson of the USDA labs in Beltsville, MD (Arnason et al. 1985). More recent studies have been able to compare the efficacy of pure azadirachtin to that of neem seed extracts, allowing for better analysis of the activity of other materials found in the extracts (Wilps et al. 1992). The most important use of purified azadirachtin in recent years has been the study of its mode of action. Recent studies have led to a much more complete picture of the activity of azadirachtin. Areas of study include target sites of azadirachtin (Rembold et al. 1989), its uptake, metabolism and excretion (Paranagama et al.1993), effects on molting (Samuels & Reynolds 1993), development (Beckage et al. 1988, Koul & Isman 1991), and inhibition of neurosecretion (Subrahmanyam et al. 1989).

The wealth of information now available about the activity of commercial formulations of neem seed extracts and azadirachtin allows for the study of non leaf-feeding stages of insects as well. Studies of Japanese beetle (*Popillia japonica* Newman) larvae (Coleoptera: Scarabaeidae) conducted at a USDA laboratory in Wooster, Ohio (Ladd 1983, Ladd et al. 1984) used neem seed kernel preparations. However, only late third instars were evaluated, and no field trials were reported. No work has been published about neem products and the Japanese beetle since that time. The purpose of this thesis is to expand the information available about the impact of neem formulations on all stages of Japanese beetle larvae in the lab and in field trials. Goals of this work include the investigation of efficacy of neem formulations for all larval stages and revision of recommendations, based in part on laboratory results, for the timing of field applications of azadirachtin.

The Neem Tree

Ancient Sanskrit writings mention the use of leaves from the neem tree to protect grains and clothing from insects (Ley et al. 1993). The tree was first described by Linnaeus in 1753, and called *Melia azadirachta* after the Persian common name: azad dirakht. The final separation and re-naming of this species to *Azadirachta indica* was done by August de Jussieu in 1830 (Ley et al. 1993). It is found in the tribe Meliae of the subfamily Melioidea in the Meliaceae. Pennington (1975) discusses the details of botanical characteristics and descriptions.

Synonyms for the scientific name and for vernacular names in Arabic, Persian, Hindi, Goan, and Sanskrit, among others, are given by many authors (Schmutterer 1990, van der Nat et al. 1991, Ascher 1993, Ley et al. 1993). The tree is evergreen, hardy and fast growing, with a round crown and medium thick bark (van der Nat et al. 1991). Its leaves are medium green, pinnate, and alternate, and grow up to 30 cm in length (Schmutterer 1990).

A native of southern Asia, as many as 14 million neem trees are estimated to grow in India alone (Ley et al. 1993). During the last 25 years, the tree has been introduced to nearly all sub-tropical countries with a suitable climate. The tree is found from sea level to about 670 m above sea level, and does best in warm to hot climates with moderate to long dry spells (Ascher 1993). It grows in poor, shallow, stony or sandy soils, including saline soils, with roots that penetrate deeply (Schmutterer 1990). Major introductions into areas with these characteristics include West Africa (where it has been established in Togo since the beginning of the century), much of sub-Saharan Africa, Saudi Arabia, and the Caribbean (Anon.1992). In the United States, the neem tree has been planted in Florida, California, and Arizona. Its major use in these introduced areas, especially in Africa, is as a shade tree or for firewood,

although it has been transplanted as well by many travelers for its medicinal and insecticidal uses (van der Nat et al. 1991; Ascher 1993). The tree does not grow in areas receiving more than 800-1,000 mm of rainfall annually, however, because it is sensitive to flooding. It is also intolerant of freezing. The tree is found on several islands in the Pacific, from Indonesia to Fiji and Mauritius

Fig. 1. A neem tree growing in its native habitat, in India.
Photo courtesy of W. R. Grace & Co. Columbia, MD.

(van der Nat et al. 1991). A lesser known species, *A. integrifolia*, that grows in moister climates, is under study (Schmutterer 1989) as a possible plant of similar insecticidal activity.

The flowers of the neem tree are white and scented, and the fruits are found on drooping panicles. The globe-shaped fruits contain one or two seeds encased in a sweet fleshy drupe. These seeds have a hard white outer cover and a brown kernel (Schmutterer 1985). Each tree produces a harvestable quantity of seeds after 10 years, yielding about 9 kg of fruit at that time, and about 19 kg after 20 years (Schmutterer 1990). The seed yield varies

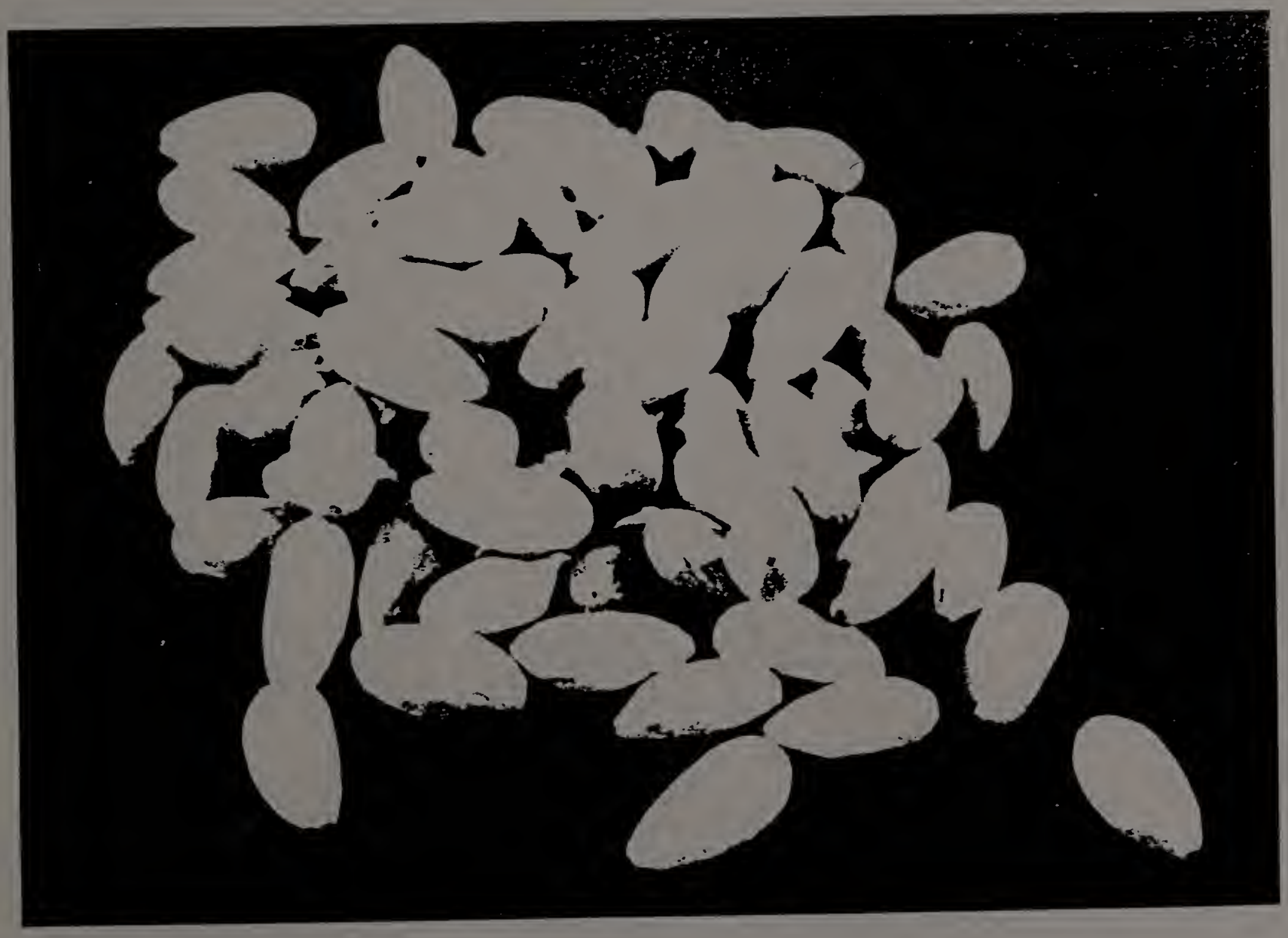


Fig. 2. Neem seed kernels ready for extraction.
Photo courtesy of W. R. Grace & Co. Columbia, MD.

in quantity and quality depending on climate. Two crops a year are reported in Togo (Schmutterer 1990), although one crop per year is more common. Trees live up to 200 years (van der Nat et al. 1991). Although neem trees are generally resistant to plant pests, the seeds are eaten by rodents, birds and bats, and controlling these mammals is necessary where the trees are grown for their seeds.

Extracts of the bark, leaves, and seeds all yield products with insecticidal properties, but the highest concentrations of these materials are found in the seeds. Ascher (1993) reports that 40 kg of fresh fruit dries to about 24 kg, the state in which the kernels are usually separated from the dried fruit. From 24 kg of fruit, about 5.5 kg of seeds are pressed into 2.5 kg of neem oil, although this varies from 17 to 50% of the fresh fruit weight and 3 kg of neem cake, a product often used as a field dressing for crops because of its high nitrogen content (Koul et al. 1990). The content of azadirachtin in seed kernels varies from 2 to 4 mg per gram of kernel, with as much as 9 mg per gram of kernel found in seeds from Senegal (Anon. 1992).

Neem Products

The constituents of *A. indica* are reviewed by van der Nat et al. (1991). The primary active ingredient, azadirachtin (Fig. 3), is found in the bark, heartwood, leaves, fruit and seeds (Ascher 1993), but is only one of nine limonoids (Anon. 1992) listed as having insecticidal activity. Among these limonoids (or tetranor- triterpenoids) are meliantriol (Fig. 4), salannin (Fig. 5) and nimbin (Fig. 6), which occur in the highest concentrations (Anon. 1992).

Meliantriol is a tetracyclic triterpene, or protolimonoid, while salannin, nimbin and azadirachtin all come from the group 4A limonoids, which are derived from the protolimonoids by way of a tetracyclic triterpenoid precursor known as tirucallol (Koul et al. 1990; van der Nat et al. 1991; Ley et al. 1993).

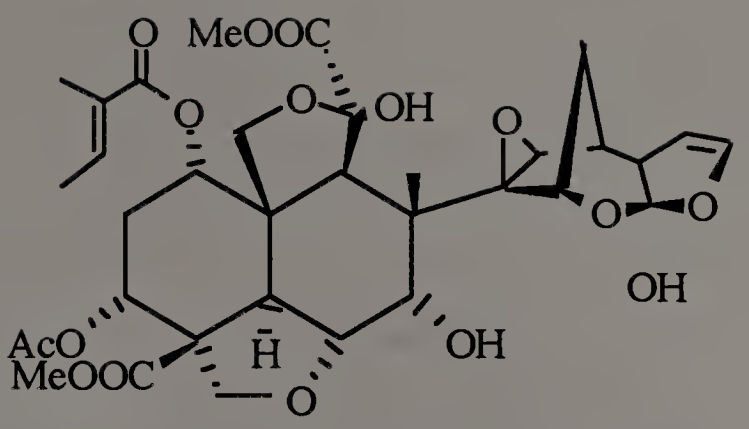


Fig. 3. The chemical structure of azadirachtin.

Many other limonoids are found in *A. indica*, but in much smaller quantities. Other constituents found in *A. indica* include diterpenes, steroids, flavonoids, flavonolglycosides, tannins, carbohydrates, proteins, alkaloids, and sulphurous compounds, which account for the odor of the oil. Also found in the triglyceride portion of the seed oil are stearic, oleic and palmitic acids (Ascher 1993).

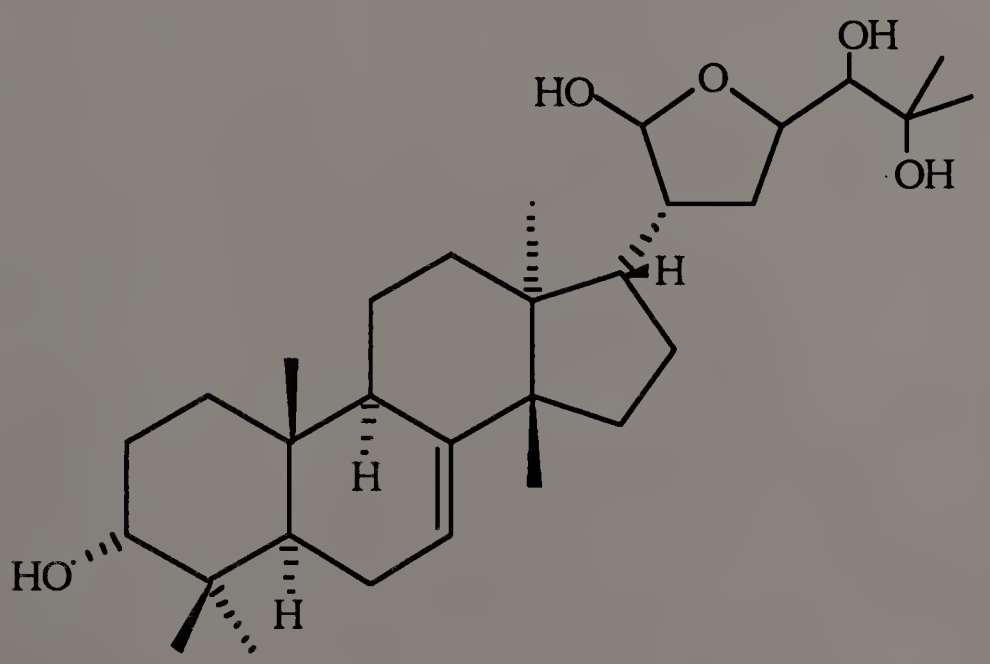


Fig. 4. The chemical structure of meliantriol.

Although the focus of this work is on the insecticidal activity of neem products, neem extracts have also been reported as having an impact against nematodes, fungi, snails, and the activity of some plant viruses. The negative impact of neem products on earthworms and on beneficial insects that prey on pest species treated with neem has been minimal in most cases (Anon. 1992).

In the tradition of Ayurvedic medicine, neem products are recommended for the cure of many illnesses (Ascher 1993). Among demonstrated medical effects, neem extracts have been shown to have anti-inflammatory, antimalarial, antipyretic, and antimicrobial effects (van der Nat et al. 1991). Extracts have recently been reported as effective against scabies and Chagas' disease (Anon. 1992). Some effectiveness has also recently been demonstrated with neem extracts used in rats as a spermicidal preparation (Prakash et al. 1988). Personal care products containing neem oil, virtually all of which are produced in India and Pakistan, include toothpastes, soaps and skin creams (Koul et al. 1989; Ascher 1993).

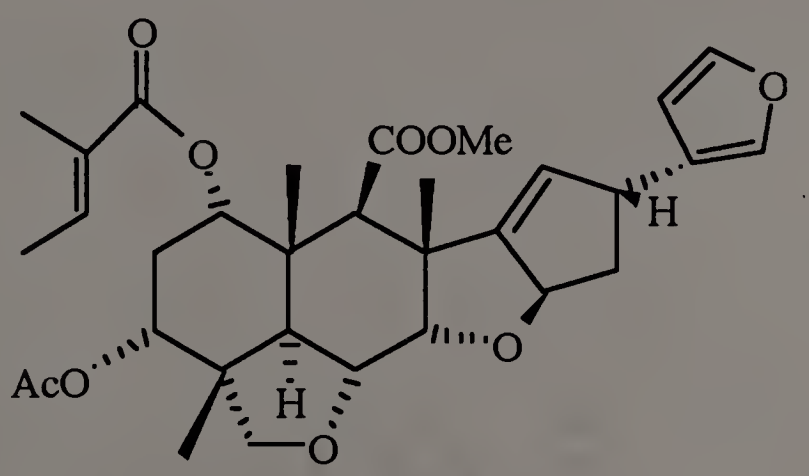


Fig. 5. The chemical structure of salannin

Neem Formulations

Neem seeds are processed in many ways. By far the simplest and most common is grinding the seeds and extracting the active ingredients with water.

In the Third World, where this method is practiced most widely, seeds in the proportion of 500 g of crushed seed kernels to 10 liters of water are steeped and used directly on crops. Depending on seed potency, between 20 and 30 kg of neem seeds are recommended to treat a hectare. This quantity of seeds can be obtained from two trees that are healthy and 20 or more years old. (Anon. 1992).

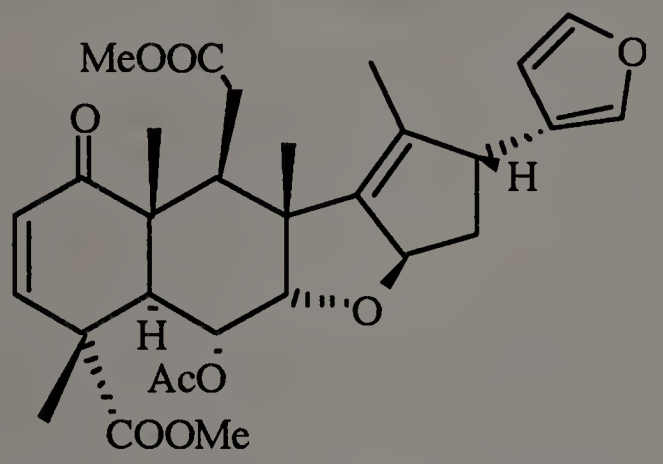


Fig. 6. The chemical structure of nimbin.

Hexane extraction removes only the neem oil, leaving the major active ingredients in the seeds, which can then be extracted with water or alcohol to produce an oil-free extract. Pentane extraction also leaves the major limonoids with the seed kernels, although this extract nonetheless has some effectiveness against spider mites and their eggs. Hexane extraction removes only the neem oil, leaving the major active ingredients in the seeds, which can then be extracted with water. Alcohol extraction is by far the most efficient extraction method, removing as much as 50 times as much of the active limonoids as does water extraction (Anon. 1992). Commercial formulations often consist of ethanolic extracts of neem seeds with some neem seed oil added to inhibit ultraviolet degradation (Ascher 1993). Some of the commercial formulations available in 1994 are listed in Table 1.

Table 1. Commercial formulations of neem seed extract

In India (Ascher 1993; Mordue & Blackwell 1993):

Wellgro and RD-Repelin,

(for cutworm and tobacco pests)

Nimbosol and Biosol

(for control of whiteflies)

all the above are produced by ITC Ltd, Andhra Pradesh

Neemguard

produced by Gharda Chemicals, Bombay

Neemark

produced by West Coast Herbochem, Bombay

In Europe (Mordue & Blackwell 1993):

Neemazal

produced by Trilolio M GmBH, Lahnau, Germany

In the U.S.A.(Anon. 1992, Ascher 1993)

Margosan-O, Bioneem, Benefit

Margosan-O is produced by W. R. Grace, Columbia, MD

Bioneem and Benefit are distributed by Ringer Corp.

Azatin, Turplex, Align

produced by Agridyne Technologies, Salt Lake City, UT

all three are distributed by O. M. Scott

In Canada (Mordue & Blackwell 1993)

Safer's ENI

produced by Safer Ltd, Victoria, B. C.

now incorporated into Ringer Corp.

Mode of Action of Neem Seed Extracts

Many effects of neem seed extracts have been reported in the scientific literature since the first reports of its efficacy were made by Leuschner (1972) and Ruscoe (1972). Ten different kinds of insecticidal effects are described by Ascher (1993). They are:

- primary antifeedant effects due to chemoreceptors present in mouthparts of particular insects
- secondary antifeedant effects due to reduction in gut motility
- delay in the development time of immature stages after treatment
- reduced uptake of food by adults due to antifeedant effects
- effects on metamorphosis
- oviposition repellency
- lowered fecundity
- egg sterility due to adult uptake
- shortening of longevity of females and / or males
- debilitating effects on fitness

These same effects have all been reported when azadirachtin alone has been tested on insects, and therefore, this one limonoid is generally assumed to be the primary active ingredient in neem extracts by most authors (Ascher 1993; Mordue & Blackwell 1993). The added impact of other limonoids, especially in cases of oral and oviposition repellency, has also been demonstrated (Schmutterer 1990). As the chemical synthesis of azadirachtin becomes more likely (Ley et al. 1993), studies of its mode of action when not combined with other limonoids found in extracts become more important. A simpler explanation of the mode of action of purified azadirachtin has been offered by Mordue & Blackwell (1993) as having effects in three areas:

- effects on taste and other chemoreceptors resulting in

antifeedancy and deterrence

- effects on ecdysteroid and juvenile hormone titre through a blockage of morphogenetic peptide hormone release, including PTTH and allatotropins
- direct effects on most other tissues studied resulting in an overall loss of fitness of the insect

Studies on *Locusta migratoria* L. by Mordue et al. (1985) and Nasruddin & Mordue (1993) on both *L. migratoria* and *S. gregaria* suggest an even simpler explanation of the many effects seen in insects subjected to azadirachtin. In both instances, considerable necrosis and senescence of midgut cells were found in insects treated with azadirachtin, due possibly to blockage of mitosis, as was found in epidermal cells and imaginal discs by Schlüter & Schulz (1984) and Schlüter (1987). This causes disruption of normal gut functions, which include feeding and nutritional uptake, the switching of the endocrine system to trigger molting, and the swallowing of air to assist ecdysis. Timmons & Reynolds (1992) also demonstrated the loss of ability by midgut cells to produce trypsin, resulting in a reduced ability to absorb nitrogen.

Much research has also been conducted on the effects of azadirachtin on the brain-corpus cardiacum complex (Rembold et al. 1989, Ascher 1993). An azadirachtin-induced blockage of normal release of trophic hormones from the corpus cardiacum causes delay and reductions in the amounts of both juvenile hormone (JH) and ecdysone from their release sites. In another study that indicated a possible mechanism for the inhibition of neurosecretory release, Banerjee & Rembold (1992) found that azadirachtin administered to *S. gregaria* and *L. migratoria* led to the accumulation of serotonin in the neuroendocrine organs and the brains of these insects. Additional studies of the movement of brain hormones and peptides in these tissues through the use of radiolabelled

cysteine and of [^{3}H] azadirachtin will lead to better understanding of these processes. Accumulation of azadirachtin in the Malpighian tubule cells is also under study (Ascher 1993). There has been significant progress in unraveling the mechanisms that account for the mode of action of azadirachtin on the cellular level as a result of the use of these and other techniques (Mordue & Blackwell 1993).

Molecular Structure and Properties of Azadirachtin

The first structure for azadirachtin was proposed by Nakanishi and his group in 1975 (Zanno et al. 1975). The correct structure (Fig. 3) was reported by Broughton et al. (1986) and by Kraus et al. (1985). Complete details were published sumultaneously by Bilton et al., Kraus et al., and Turner et al. in the same issue of Tetrahedron in 1987. Azadirachtin is a highly oxidized limonoid with many reactive groups in close proximity (Mordue & Blackwell 1993). Its biosynthesis is thought to involve the steroid tirrucallol (Ley et al. 1993), although tirucallol (Fig. 7) has never been isolated from azadirachtin. On the material safety data sheet provided by Agridyne Technologies, azadirachtin is characterized as a tetranortriterpenoid with a molecular weight of 720, a formula of $C_{35}H_{44}O_{16}$ and a specific gravity in water of 1.060. Its acute oral LD_{50} (dose at which 50% of the treated group is killed) is 3540 mg/kg (rats) with no known chronic effects.

Aldhouse (1992) reports that the activity of the different parts of the molecule have been characterized by Ley and his colleagues at the Imperial College of London. The insecticidal left portion of the molecule, called the decalin fragment, is responsible for the disruption of insect growth and development, while the right portion, or hydroxy furan fragment, is responsible for antifeedant activity (Fig. 8). Both fragments have been synthesized by these

chemists (Ley et al. 1993), but joining them has not as yet been accomplished. Aldhous suggests that a goal of commercial producers is to intensify research on these chemical synthesis studies in order to develop azadirachtin-based insecticides that can be patented, in contrast to neem extracts that include azadirachtin A, whose formula was published so long ago that it cannot be patented.

Some work on the alteration of the azadirachtin molecule is already under way. Ley and his group have been able to provide some resistance of azadirachtin to acidic degradation by alkylating a hydroxyl group, and providing improved resistance to ULV degradation by hydrogenating all the molecule's double bonds (Aldhous 1992). Similar efforts are underway at Agridyne

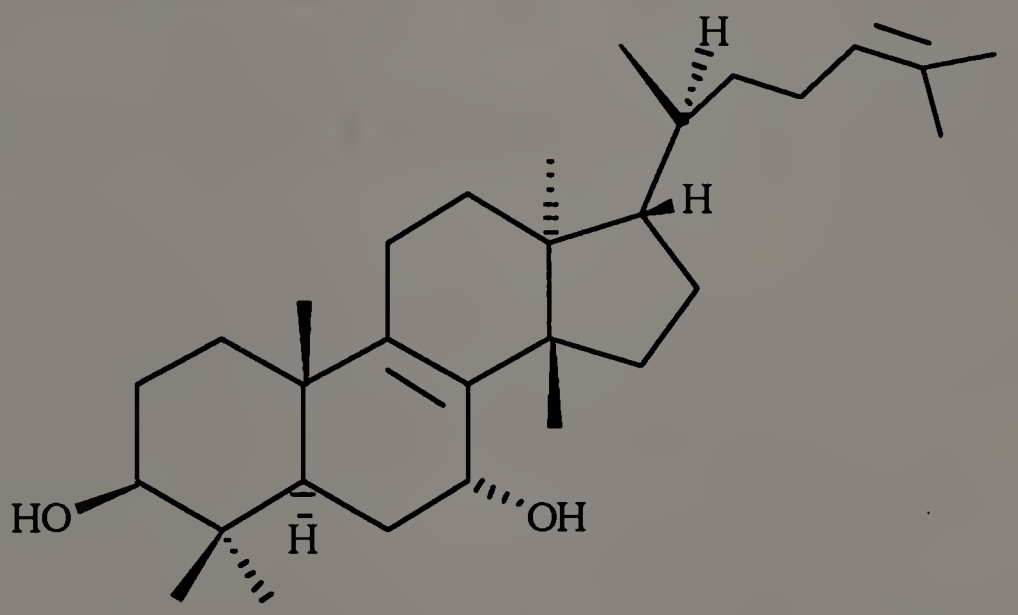


Fig.7. The chemical structure of tirucallol.

Technologies, where their development of tetrahydroazadirachtin features saturation at the 2' and 3' sites of the molecule. This procedure results in less than 10% ULV degradation in 400 hours, and is projected to provide a stable formulation that remains on leaves to control leaf feeding insects 2 or 3 times longer than azadirachtin does (Immaraju 1992).

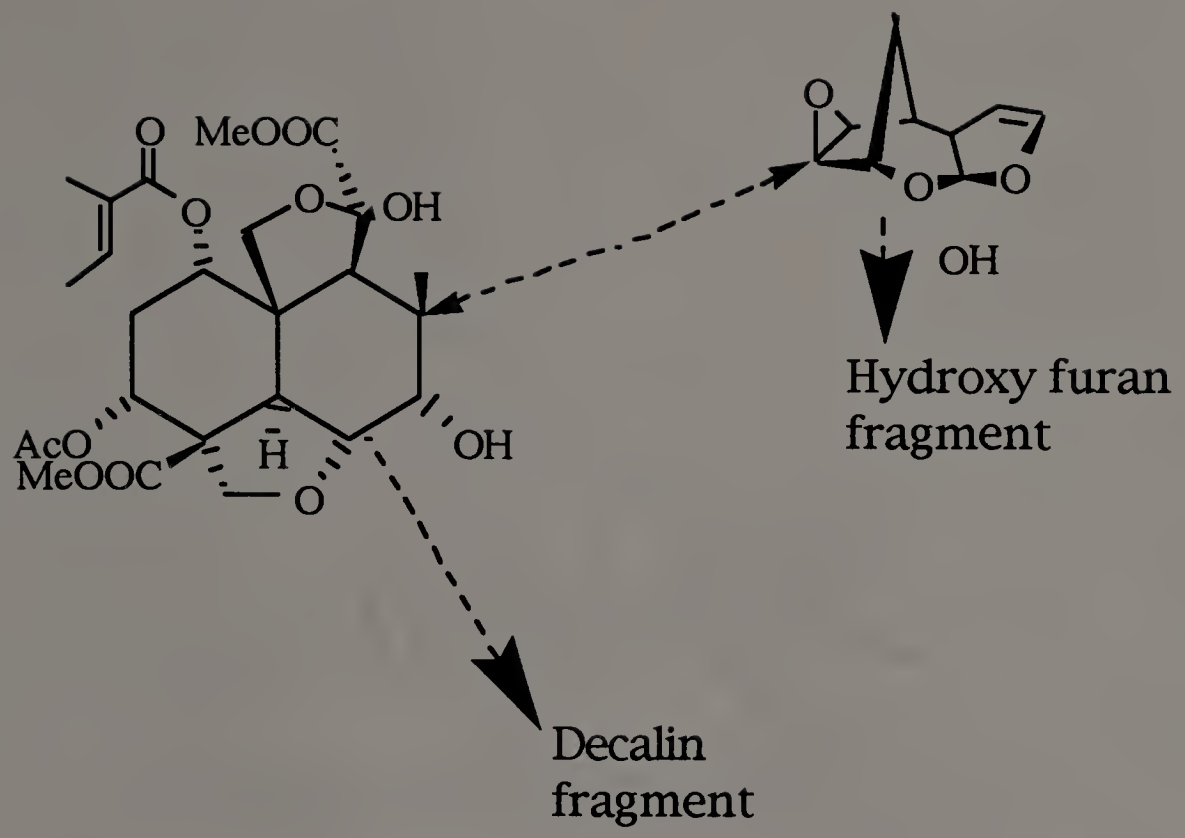


Fig. 8. The two fragments of the azadirachtin molecule.

Many isomers of azadirachtin have been elucidated along with other limonoids extracted from neem products. Azadirachtin A (Fig. 9), (Rembold 1989) is the most common isomer, accounting for most of the total, followed by azadirachtin B (Fig. 10), or 3-tigloylazadirachtol, which often occurs in concentrations up to 20% of azadirachtin A (Mordue & Blackwell 1993). Other isomers, called azadirachtins C through I, as well as the compounds vepaol, isoepaol and marrangin have been reported as well, but at much lower concentrations, generally in the range of one or two orders of magnitude below azadirachtins A and B (Ley et al. 1993). Only azadirachtins A and B can be obtained in sufficient amounts at present for all but the smallest laboratory investigations. As the synthetic pathways of the other isomers are found and produced in sufficient amounts, evaluation of their usefulness as insecticides will also become possible.

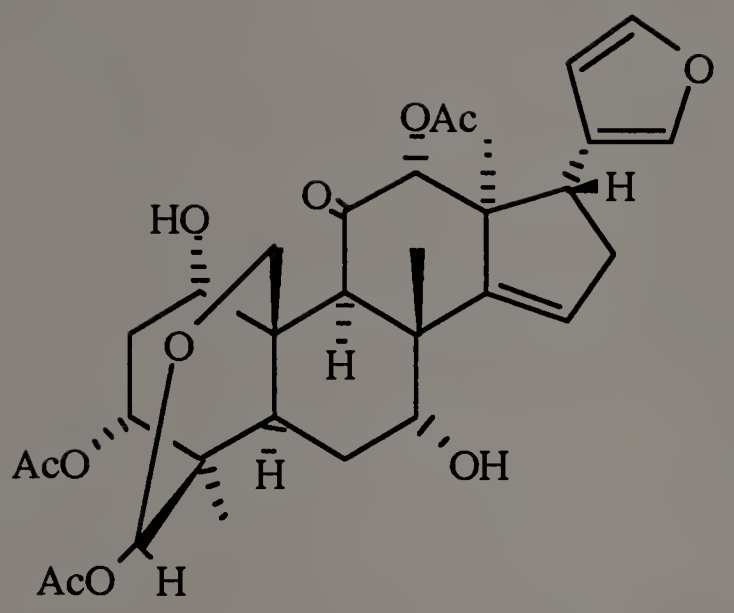


Fig. 9. The chemical structure of azadirachtin A.

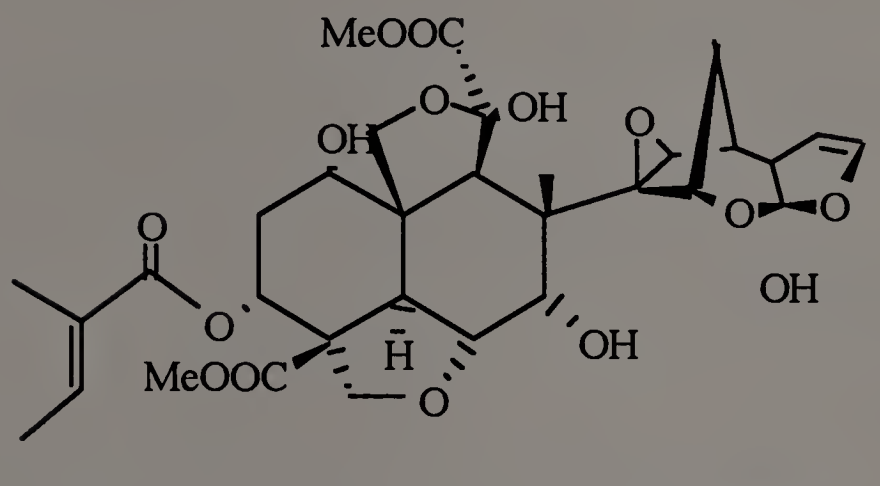


Fig. 10. The chemical structure of azadirachtin B.

Control of the Japanese Beetle with Insect Growth Regulators

Japanese beetles, along with other white grubs, are the most damaging insects of turfgrass in the northeastern United States and southeastern Canada (Tashiro 1987, Potter & Braman 1991). A univoltine insect throughout all but the coldest parts of its range, the Japanese beetle spends 9 months of the year as a third instar, and increases in weight nearly a hundred-fold between hatch and pupation (Fleming 1972). Unlike other white grubs, Japanese beetles adults also cause serious damage to over 300 species of ornamental and indigenous plants (Potter & Braman 1991). It is the combination of larval destruction of turf and adult voraciousness that makes this species particularly widespread and damaging (Tashiro 1987).

The beetle was introduced in the United States in 1916, probably in the soil of potted nursery stock, from Japan. It is found on the four main islands in Japan, especially in the northernmost island of Hokkaido, which shares a climate similar to that of New England. It has little importance as a pest in Japan because of many natural enemies (Fleming 1972). Its current distribution includes all states east of the Mississippi (Potter & Braman 1991) and parts of the Midwest (Tashiro 1987).

Ever since the elimination of cyclodiene and organochloride insecticides for soil treatments in the late 1960's and early 1970's, acceptable control of white grubs on lawns and golf greens has been achieved using other traditional cholinesterase-inhibiting insecticides (Tashiro 1987) or synthetic pyrethroids (Potter & Braman 1991). As pesticide labels and materials have become more restricted, however, interest in more biorational approaches to grub control has increased. Several national companies interested in developing these newer pesticides have begun to investigate the use of insect growth regulators and evaluate them for label registration. Some of the research in this thesis is supported by companies interested in such evaluations.

Although Potter and Braman (1991) mention no commercially registered insect growth regulators for control of turf insects as recently as 1991, results of field trials of neem formulations and a nonsteroidal insect growth regulator (Potter & Spicer 1993) have appeared in the Insecticide and Acaricide Tests for 1991- 1993 (Baxendale & Weinhold 1991, Spicer et al. 1992a & b, Vittum et al. 1992, Weaver & Daniels 1992, Heller & Walker 1993 a & b, Power et al. 1993, Smitley & Davis 1993). Much remains to be learned about optimal uses of insect growth regulators to control pests in turf situations. It is hoped that the work undertaken in this thesis will prove a useful addition to this overall understanding.

CHAPTER II
EVALUATION OF REARING TECHNIQUES FOR JAPANESE BEETLES

Life History of the Japanese beetle

The biology of the Japanese beetle has been described in the United States by Fleming (1972) and Tashiro (1987), and in Massachusetts by Vittum (1986). This insect is univoltine, with varying proportions in the northern range taking two years to complete a generation. In Massachusetts, for example, Vittum (1986) found that 10% of larvae sampled in the field required two years to reach adulthood. In the Northeast, the adults emerge in mid to late June and survive an average of 30 to 45 days after emergence (Fleming 1972). Their activity is diurnal, and beetles are most active on sunny days when the temperature range is 21 - 35^{o}C and the relative humidity is 60% or greater (Fleming 1972). Beetle emergence patterns are also strongly affected by rainfall (Morrill & Dobson 1978). Callow adults remain in the soil from 2 to 14 days while their wings unfold and harden and their reproductive organs mature. The peak of male emergence preceeds that of female emergence by as much as a week to 10 days (Vittum 1986). Adults construct a tunnel from the pupal chamber to the soil surface and return to it, at least initially, whenever the weather is too cool, too warm, or too wet for nuptial flights or feeding activity (Fleming 1972).

Unmated females produce a sex pheromone, and once fertilized, they return to the soil to oviposit. Females continue to mate after each cycle of oviposition, usually with different males (Fleming 1972). Mated females burrow

5 -10 cm into moist soil and deposit 1 - 3 eggs in adjacent cells. Peak oviposition occurs in late July in Virginia, early August in southern New Jersey, and late August in New Hampshire (Fleming 1972, Vittum 1986). Females lay from 30 to 50 eggs throughout their lifetime (Régnière et al. 1981c) and may burrow into the soil as many as 16 times during that period to deposit eggs (Fleming 1972).

Eggs eclose after about two weeks (Tashiro 1987). Egg survival is dependent on soil moisture, as newly laid eggs must absorb sufficient amounts of water for embryonic development to proceed. Delays in egg hatch have been reported by Régnière et al. (1981c) under both saturated and extremely dry soil conditions (particularly with finer textured soils). Mean hatch time reported by Régnière et al. (1981c) was 12.498 $\pm$ 0.001 days. First instars form a small cell in which they remain, feeding on fine rootlets, for two to three weeks. Second instars molt after three to four weeks. A few second and many more third instars [average yearly ratio about 10:90 (Vittum1986)] continue to feed throughout autumn before moving downwards in the soil to overwinter (Tashiro 1987).

As soil temperatures decline in the fall, beginning at 15^{o} C and culminating when average soil temperatures reach 10^{o} C, the larvae (both second and third instars) feed less and move downward in the soil profile, remaining 5 - 25 cm below the surface. Activity resumes in the spring when soil temperatures again climb to 10^{o} C, when larvae return to within 5 cm of the surface. They feed for 3 - 4 weeks at the soil/thatch interface and then move down 5 - 10 cm to form a pupal cell and begin pupation. Pupation lasts 8 - 20 days, when the callow adults emerge, completing the cycle (Tashiro 1987). Development time for all stages is temperature dependent (Fleming 1972, Régnière et al. 1981b).

Larval behavior is instar dependent. Villani & Nyrop (1991), in their studies of larval behavior, report very little movement among neonate larvae. They describe a strong random character to the host seeking of neonates for the first 72 h of behavior tests they conducted, and larval arrestment in sod only after that time. That neonates move relatively little is explained in part by the size of the insect in relation to soil pore size (Villani & Wright 1990), with smaller larvae less able to move easily around soil particles than larger larvae. Second instars exhibit a strong attraction to sod, as well as improved mobility, and pronounced arrestment at the thatch/root layer, while third instars move down as well as towards sod as the amount of roots they consume increases (Villani & Nyrop 1991). In addition, larvae respond to changes in soil moisture and temperature, moving downward to avoid dry and particularly warm (>27^{o} C) soil temperatures in the summer, and in response to falling soil temperatures in autumn (Villani & Wright 1988).

Populations of larvae sampled in the field tend to exhibit a negative binomial (i.e., clumped) distribution (Régnière et al. 1981b, Ng et al. 1983), although measures of aggregation are lower in spring populations (Ng et al. 1983) after the surviving overwintered larvae return to the surface to feed. The clumped distribution of larvae is related to female ovipositional choice. Females exhibit a strong preference for oviposition in moist soils of sandy loam to silt loam textures. Females normally do not oviposit in dry soils of any texture (Régnière et al. 1979). Levels of oviposition and mortality of both larvae and adults have been related by many authors to rainfall patterns (Hawley 1949, Fleming 1976, Régnière et al. 1979).

Choice of oviposition site apparently is related to proximity to preferred adult food sources (Fleming 1972). A report by Bohlen & Barrett (1990) indicated that in field crop situations, movement patterns of adult Japanese

beetles can be influenced by interspersing preferred foods such as soybeans with nonpreferred ones such as tall sorghum. Several factors can influence ovipositional choice, including soil moisture, texture, temperature and mowing heights (Régnière et al. 1981c). How females select sites for oviposition, whether ovipositional kairomones play any part in the resulting clumped densities of eggs, and if individual females return repeatedly to the same site to oviposit are unreported. Whether females return to areas adjacent to those from which they emerged to oviposit is also unknown.

Laboratory Rearing of Japanese beetles

Introduction

Information regarding laboratory rearing of Japanese beetle grubs was most recently published in 1985 (Goonewardene 1985). Since then, rearing of insects in general has been affected by changes such as the improvement in types and availability of rearing supplies, particularly substitution of individual 30 ml cups to store insects for the metal tins used to store groups of insects, and more sophisticated environmental chambers. No refinements of methods of colony maintenance have been published since 1985.

Over 7,500 eggs were harvested in 1992 from adults trapped and processed in a rearing facility in Amherst using methods developed in the lab of Dr. Michael G. Villani of the NYSAES (unpublished data) in Geneva, NY. Fewer eggs (3,500 - 4,000) were harvested during 1993 because of extreme drought conditions in July and August that strongly affected adult beetle survival and fecundity. In this section, I describe the techniques used to collect adults and establish a colony of larvae from their eggs, including survival of these larvae over periods ranging from 1 - 14 weeks in an environmental chamber.

Materials and Methods

Adult beetles were collected in USDA Japanese beetle traps baited with commercially available lures [phenethyl propionate, eugenol and geraniol in a ratio of 3:7:3 (Alm et al. 1994)], placed in containers with moist sponges to provide water and transported to the lab within a day of capture. At the lab, beetles were first placed in the refrigerator at $5 \pm 1^{o}C$ to slow their activity. Beetles were then separated by sex, and placed in 17 x 17 x 8 cm^3 screened plastic boxes filled to within 3 cm of the top with a moist (12 - 15 % moisture by weight) sharp builder's sand/screened peat mixture [2:3 (v:v)] at the rate of 100 females and 10 males per box. Leaves of preferred foods (buckthorn, blackberry, rose, linden and others) in 5 ml water tubes were added on top of the sand and the beetles were held for 3 - 5 days, with food added as needed, in an environmental chamber at 23^{o} C and a 12:12 (L:D) h photophase.

After this time, the adults were removed and the sand/peat mixture was sifted with a number 10 or 12 mesh screen to remove adults and large debris. Live beetles were placed in a new box of oviposition mix and leaves, combined with newly collected beetles to bring the numbers back to 110, and returned to the environmental chamber. Dead beetles were discarded. The screened oviposition mix was then carefully re-screened to remove the eggs deposited. Eggs older than 3 - 4 days were easier to find and pick up (using soft aluminum forceps) than were newly laid eggs, and in cases where the eggs were found to be small, the oviposition mix was sometimes moistened as needed and returned to the environmental chamber until the eggs had developed to a point where they could be handled more easily. Eggs harvested in this way were placed in a container with a very fine mesh bottom and washed with cool water. The rinsed eggs were then wrapped overnight (or up to 3 days) in moist paper towels inside a covered petri dish to allow them to swell. They were then

transferred to petri dishes of the same moist sand/peat mixture and returned to the environmental chamber.

The eggs were transferred when they reached the 'mandible egg' stage (mandibles become visible through the chorion of the egg two or three days prior to hatch), either individually in 30 ml plastic cups with lids or as groups in 32 x 23 x 9 cm^3 plastic tubs with lids containing a soil mix of fine sandy loam and screened peat moss [8:1 (w:w)] seeded with Agway 'Shady Lawn' grass seed. Soil moisture was kept between 9 -12% by weight and the insects were checked weekly to add seeds and moisture as needed.

Fig. 11. Japanese beetle larva in a 30 ml plastic cup of soil
P. J. Vittum photo

Larvae collected from the field were dug and placed directly into plastic tubs filled to within 5 cm of the top with moist loam (Hadley silt loam). In the

spring of 1992, field collected insects used for the diet test were transferred to trays of cups held in the lab at ambient temperatures and light sources. During 1991 and 1992, larvae harvested from the field in the summer were placed in 32 x 23 x 9 cm tubs of soil mix and seeds at the rate of approximately 200/box and stored in an environmental chamber at 23 $\pm$ 2^{o} C and a 12:12 (L:D) h photophase until needed for bioassays.

In 1993, to avoid mortality due to wounding and cannibalism, after field dug larvae were returned to the lab, they were placed individually in 30 ml cups filled 80% with seeds and the soil / peat mixture. All larvae failing to dig into the soil within 10 minutes were discarded. Cups of larvae were sorted using a random number table into test groups and held overnight. These groups were used for subsequent tests. Any dead larvae found in these groups when they were transferred to clean cups containg the test soils were replaced from a group of insects sorted into a reserve group. All extra larvae not used immediately were provided grass seeds and stored in trays in the environmental chamber until needed for subsequent studies.

A summary of the development times and survival of insects from the control (untreated) groups from different bioassays conducted from 1992 to 1994 appears in Table 2. All but one set of insects listed in Table 2 were reared in an environmental chamber at 23^{o} C and 75% relative humidity (RH) on a 12:12 (L:D) h photophase. The one exception was reared in the laboratory at ambient temperatures and no set light schedule. Numbers of insects per treatment in each bioassay and details of how these assays were prepared are found in Chapter III.

Results

Laboratory bioassays were kept initially for the same duration as was recommended in the instruction material provided by Agridyne Technologies with its neem formulation and used to time sampling for the formulations in field trials, and as a result, insects reared during 1992 were not always held to adult emergence. Once it became apparent during 1992 that treated insects continued to respond to the neem formulations until adult emergence, subsequent tests were maintained until all possible larvae had completed development or died. Unfortunately, with the exception of a few individuals in tests of eggs and second instars, insects in the control treatments of bioassays conducted during the summer of 1993 did not alway survive to the adult stage, but the tests were observed until this outcome became apparent. Insect survival was better in treatments of 0.05 or 0.1 ppm, and these insects were used instead as the indicators of when to end the tests in 1993.

During 1993, no larva remaining more than two weeks beyond the peak of adult emergence developed into an adult. Often the last few insects remaining in a test were infected with a milky disease [apparently either *Bacillus popilliae* Dutky or *B. p. var. lentimorbus* Dutky (Hanula & Andreadis 1988)] whose symptoms were not apparent until the end of the assay. Third instars used for assays in the spring of 1993 and 1994 averaged nearly the same amount of time to complete development once in the chamber (around 30 days) and emerge as adults. Mean survival at the onset of adult emergence, when the test ended, was 40% in 1993. Mean survival was 55% in 1994, when insects were kept until all adults had emerged.

Insects reared from summer harvested insects or those reared from eggs varied in their survival rates, ranging from 7 - 52% when survival to adult emergence was measured. In particular, those in the control treatments of

Table 2. Development times of insects in the control treatments of bioassays held at 23°C and 75% RH on a 12:12 (L:D) h photophase

Insects collected in the spring after overwintering in the field

instar	year	start date	no. of days in chamber	mean no. of days to adult emergence	reared to adult stage	mean % survival
third	1992[a]	5/8	34	34	yes[b]	not comparable
third	1993	6/4	60	32	yes	40
third	1994	4/25	52	30	yes	55

Lab-reared insects and those dug in the field in the summer

instar	year	start date	no. of days in chamber	mean no. of days to adult emergence	reared to adult stage	mean % survival
egg	1993	8/13	188	181	yes	10
first	1992	8/26	27	kept 27 days	no	not comparable
first	1993	8/13	189	no survivors	yes	0
second	1992	8/31	151	138	yes[b]	52
second	1993	9/7	164	163	yes	7
third	1992	8/31	112	66	yes[b]	24
third	1993	9/20	135	no survivors	yes	0

a. third instars collected in the spring of 1992 reared in the lab at ambient temperatures

b. insects kept in environmental chamber up to the onset of adult emergence, but not until all larvae had pupated

bioassays of first instars suffered the highest mortality (0 survived to the adult stage in 1993), probably in part because of sensitivity to handling. Simply placing 'mandible eggs' or second instars in similar conditions produced much higher survival rates (10 - 52%). Populations of third instars harvested in the field varied considerably in their survival rates (0 - 24%).

In all cases where larvae were stored together in the large containers for more than a few days, larval numbers declined steadily. Because of this, field dug insects were not stored in tubs for more than 48 h after 1992, but were transferred to individual cups and maintained in trays before use in bioassays. Although a small decline in numbers of larvae stored individually also occurred, insect survival in 30 ml cups remained above 60% after the first month post-harvest on average, declining to the survival ranges listed in Table 2 at adult emergence. Maintenance of the 30 ml cups at about 10% soil moisture provided the best overall survival during bioassays.

Long periods of dry weather during June and July of 1993 had a strong impact on the vigor and survival of the Japanese beetle population that year, which is reflected in the results shown in Table 2. Results of insect rearing conducted in 1992 and 1994 are more representative of insects from populations harvested during normal weather conditions. Populations of larvae harvested during the spring appeared to be highly synchronized in their emergence times, while summer populations were less so. Insects reared from eggs and kept in the environmental chamber until adult emergence took much longer proportionally to complete development than did insects dug in the summer as second or third instars and reared in the environmental chamber.

Discussion

Field harvested insects placed into treatments as second or third instars had a shorter development time than did second or third instars reared from eggs in an environmental chamber at a constant 23° C. Fleming (1972) observed that larvae tolerated temperatures in the lab up to 27.5° C. Goonewardene & Zepp (1970) reared younger larvae at 29° C and 80% RH and third instars (1969) at 26.5° C and the same RH. Perhaps rearing early instars at these higher temperatures would accelerate development in laboratory-reared populations in the future.

Maintaining populations for periods longer than a month or six weeks remained difficult at rates above 30% survival in my studies. Goonewardene & Zepp (1970) reported per cent survival rates ranging from a mean of 62.5% at 6 days after storage to a mean of 27.4% at 90 days after storage. They list possible contamination of the colony with the bacteria, *Serratia marcescens* Bizio, as one explanation of these outcomes. In my efforts to rear beetles, I tried to eliminate mortality due to competition (for all stages) and due to handling (first instars). No pattern was seen that distinguished survival of larvae reared from laboratory colonies as compared to those harvested from field populations. Such a difference might indicate some success in eliminating mortality due to microorganisms. Sterilized soil was not used, as field dug larvae placed in sterilized soil contaminate rearing media with the first evacuation of their hindguts, which may contain numerous microorganisms (Hanula & Andreadis 1988). Until a way is found to eliminate these microorganisms from field dug insects, or prevent them in laboratory-reared ones, survival rates will probably remain in the ranges cited by Goonewardene & Zepp (1970). Further study is needed to address the limitations of rearing Japanese beetles for laboratory bioassays.

Evaluation of the New Zealand Grass Grub Diet

Introduction

Larvae reared on soil and grass seeds suffer from some background mortality due to contamination from the soil or the insects themselves, which are brought in from the field with an array of microorganisms in their digestive systems (Hanula & Andreadis 1988). The use of a clean food source, particularly one that could have known concentrations of azadirachtin or other materials incorporated in it, was an attractive idea for colony maintenance. I hoped to compare the survival of Japanese beetle larvae reared on this diet with that of larvae reared in soil with grass seeds, in order to see if the diet had merit as a possible substitute. Two preliminary tests were conducted to assess whether size of cup or placement of the insect outside of its soil environment were critical. A third diet test compared diet alone or diet in peat moss with the "traditional" soil and seeds method.

Materials and Methods

Third instar Japanese beetles collected from a golf course in Amherst, MA, during April of 1992 were used to evaluate the artificial diet. A one liter recipe of the New Zealand Grass Grub Diet was prepared (Wigley & Dhana 1992) (Table 3). For the two preliminary tests, square cubes of finished diet approximately 5 mm per side were placed in either 30 ml or 60 ml cups with soil [eight parts fine sandy loam: one part sifted peat moss, (w:w)] or in cups without soil. The cups were capped and placed in trays at ambient temperatures, which were recorded with an minimun-maximum thermometer. For the third diet test, 80 insects per treatment were placed, one per cup, in 30 ml cups containing diet alone, diet with moistened and sifted peat moss, or the same soil mix without

diet but with added grass seed. Cups were placed in trays (Fig. 12) randomly by treatment and were stored in the same manner described for the two preliminary tests, and each cup was inspected every 2 - 3 days, when the number of survivors present was recorded. In addition, each insect was weighed on a Mettler PE 360 scale at every inspection.

The diet is described in Table 3. Part A was autoclaved, blended and allowed to cool to 70 ^{o} C, and combined with the ingredients in part B and the vitamin mix. Sorbic acid was omitted from the diet based on recommendations in Dunkel & Read (1991), to minimize growth regulator effects when using sorbic acid in bioassays conducted with artificial diets. The pH of the diet was adjusted to 4.6 with hydrochloric acid. The low pH was recommended for optimal effect of the anti-microbials. The diet was then poured into clean plastic containers and refrigerated until needed.

Results of the third diet test were analyzed using Super-Anova software (Abacus Concepts Inc., Berkley, CA) for descriptive statistics and one-way ANOVA (one-tailed test) for differences among treatments.

Results

Larval survival in the first two preliminary tests is shown in Tables 4 and 5. As can be seen in Table 4, survival after 48 days was twice as high in cups with soil added as it was in cups without soil. Test populations were small, however, and showed no clear distinctions in survival as a function of cup size.The 30 ml cup size was used in subsequent tests because these trays were easier to handle. In the second diet test shown in Table 5, distinctions between cups containing soil and seeds from those with diet alone were less clear. This test was conducted for a shorter period (39 days) that may not have been long enough for differences to appear. Because the number of insects in the

Table 3. The New Zealand Grass Grub Diet

Part A	
Lima beans, soaked overnight and drained	100 g
Casein	10 g
Water	900 ml
Sucrose	30 g
Glucose	3 g
Trehalose [a]	3 g
Agar	40 g
Potassium acetate [b]	750 mg
Sodium proprionate [b]	750 mg
Sorbic acid [c]	1.25 g
Part B	
Inositol	500 mg
Penicillin	350 mg
Streptomycin	350 mg
50/50 linseed (flaxseed)/wheatgerm oil [b]	1 ml
10% formaldehyde [b]	4 ml
Autoclaved sieved peat	100 g
Vitamins	
Ascorbic acid	3 g
Thiamine hydrochloride	2.5 mg
Niacin	10 mg
Pyridoxine hydrochloride	2.5 mg
Folic acid	2.5 mg
Calcium pantothenate	30 mg
Biotin	200 ug
Vitamin B12	40 ug
Riboflavin	500 ug

[a] Trehalose for neonate diet only, used as a feeding stimulant

[b] Omit for neonates

[c] Sorbic acid was omitted

Table 4. Preliminary diet test 1, conducted in spring, 1992, evaluating the effect of cup size and presence of soil on larval survival. Field dug Japanese beetle third instars were reared for 48 days on the New Zealand grass grub diet at ambient temperatures that ranged from 21 - 30° C.

treatment	N	final no. of adults	total dead	% survival
no soil				
30 ml cups	18	2	15	17
60 ml cups	18	1	14	22
soil added				
30 ml cups	17	5	11[a]	39
60 ml cups	17	4	8[a]	56

[a]One dead as a pupa

Table 5. Preliminary diet test 2, conducted in spring, 1992, evaluating the effect of added soil on larval survivorship. Field dug Japanese beetle third instars were reared for 39 days on the New Zealand grass grub diet at ambient temperatures that ranged from 21 - 30^{o} C.

treatment	N	final no. of adults	total dead	number remaining	% survival
no soil	30	1	8	14	47
soil added	30	1	12	17	57

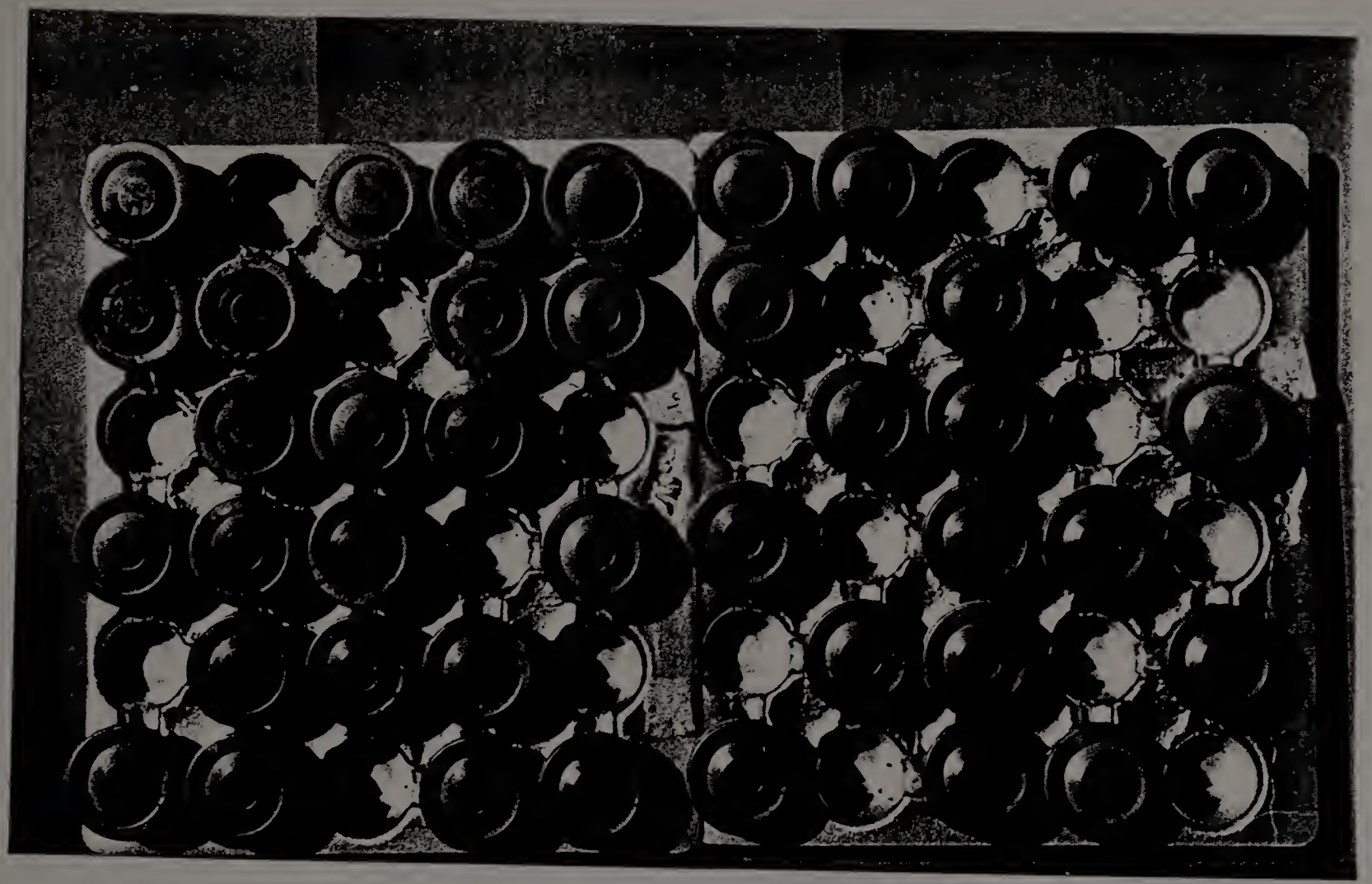


Fig. 12. Trays of larvae showing fresh weights on lids.
P. J. Vittum photo

preliminary tests were small, the tests were not continued and no statistical analysis was performed.

The remaining field harvested larvae were utilized in the third test, which contained larger numbers of insects for each of three treatments. Mean fresh weights and larval survival for each treatment in the third test are shown in Table 6. Although the treatments were sorted into trays in a random distribution (Fig.12), the insects were not first sorted randomly into treatment cups. The distribution of insects into test groups without the use of a random number table

Table 6. Survival and weight gain of Japanese beetle third instars, spring 1992, reared for 24 days on artificial diet or soil and grass seeds at ambient temperatures that ranged from 21 - 30° C.

A. Diet alone

date	temp. range °C	larval mean (± SE) fresh weight (mg)	no. alive[a]	% survival
5/08	22-27	238.0 ± 3.4	80	100
5/12	21-26	236.6 ± 3.4	80	100
5/18	21-27	241.7 ± 3.5	71	89
5/23	24-30	240.0 ± 3.6	67	84
5/26	22-27	240.2 ± 3.9	62	76
5/29	21-29	241.5 ± 4.0	57	71
6/01	22-27	241.2 ± 5.0	49	61

No. pupated by 6/01/92: 0; change in mean fresh wt.: + 3.2 mg

B. Diet and peat moss

date	temp. range °C	larval mean (± SE) fresh weight (mg)	no. alive	% survival
5/08	22-27	271.3 + 4.6	80	100
5/12	21-26	274.9 ± 4.4	78	98
5/18	21-27	272.7 ± 4.7	72	90
5/23	24-30	271.3 ± 4.6	70	88
5/26	22-27	273.0 ± 4.7	69	86
5/29	21-29	274.5 ± 6.0	67	84
6/01	22-27	275.3 ± 5.0	63	79

No. pupated by 6/01/92: 6; change in mean fresh wt.: + 4 mg

C. Soil and grass seeds

date	temp. range °C	larval mean (± SE) fresh weight (mg)	no. alive	% survival
5/08	22-27	249.8 ± 4.1	79	100
5/12	21-26	251.3 ± 3.8	79	100
5/18	21-27	252.7 ± 3.9	76	96
5/23	24-30	251.7 ± 4.0	66	84
5/26	22-27	252.6 ± 4.0	64	81
5/29	21-29	253.3 ± 4.4	64	81
6/01	22-27	253.9 ± 4.8	60	76

No. pupated by 6/01/92: 12; change in mean fresh wt.: + 4.1 mg

[a]includes pupae; test concluded at onset of adult emergence

resulted in beginning mean weights for each group that were significantly different ($P < 0.0001$). Analysis of the change in mean weight for each group from the start of the test until its end 24 days later showed an increase in mean weight of 3.2, 4, and 4.1 mg for treatments A, B, and C respectively (Table 6). These increases represented a change in mean weight of 1.3, 1.4, and 1.6%, respectively among those insects surviving at the end of the test. These changes were too small an increment for a comparison of significant differences among the three groups to be attempted. Survival of insects reared on the grass grub diet and peat was similar to that of insects reared in cups containing soil and seeds alone (79 and 76%). Survival of insects in cups with cubes of diet alone was only 61%. No insects in the diet-alone treatment pupated, while six individuals pupated in the "diet and peat" treatment, and twelve pupated in the "soil and seeds" treatment.

The cost to prepare a liter of diet was very close to $10.00. Dr. Chih Yin, a professor of insect physiology in the Department of Entomology at the University of Massachusetts, recommended trying a general insect vitamin such as the Hoffman-LaRoche premix (Yin & Peng 1981) in substitution for the vitamins listed in the grass grub diet, because the premix contains the same ingredients and cost less than do the ingredients ordered individually. Since the vitamins had already been ordered, we followed the specifications for the original diet, but calculated that using the premixed vitamins would reduce the cost of materials nearly 50%. We found it was possible to prepare the agar mix in a microwave, which was easier to control than an autoclave. The mix was microwaved on full power for three minutes, then stirred. This process was repeated three to five times until the mixture thickened.

Discussion

In the test of the grass grub diet, both increases in mean weight and larval survival to the pupal stage were used as outcomes to measure the success of each treatment. Larval survival to the adult stage would have been a better criterion, as it accounts for mortalities at the larva to pupa and pupa to adult molts that occur in less fit individuals. Increases in mean weight for each treatment were smaller than expected and provided little useful information. In addition, loss of some larvae to mortality or pupation changed the composition of each group, especially at the end of the test, and may have made comparison by differences in weight gain more difficult to assess. In any case, results from the three tests led me to continue using the soil and seeds method of rearing grubs for the bioassays described in Chapter III.

The small increases in weight for each treatment group may have been a normal characteristic of third instars nearing pupation, but it was also possible that insects in the 'diet alone' treatment were not ingesting much of the diet. Several changes in the diet, such as the substitution of liophilized grass roots for peat moss, substitution of a vitamin pre-mix for the vitamins, and methyl paraben for other preservatives could be tried to improve the diet to make it more attractive for Japanese beetles, but it would be necessary to evaluate third instars on the diet for a longer period of time than was the case for this test to be certain. Until such a study can be conducted, rearing Japanese beetles with the soil and seeds method described in this study remains the most acceptable method, with survival rates comparable to or better than those for insects reared on the grass grub diet. The raising of disease-free laboratory populations of larvae is a technique that remains to be developed, but which should go a long way towards improving bioassays with Japanese beetles.

CHAPTER III

BIOASSAYS OF AZADIRACHTIN ON JAPANESE BEETLES

Bioassays Using Topical Application

Introduction

The use of extracts of the seeds of the neem plant, *Azadirachta indica*, for control of leaf-feeding arthropod pests has been investigated extensively during the last twenty years (Warthen, 1989). More recently, the efficacy of neem products has been studied in relation to soil-dwelling insects (Stark 1992). Ladd (1983) evaluated the topical effect of azadirachtin, the most active of the components of neem extract, on late third instar Japanese beetles. Spring-harvested third instars are often used for bioassays involving traditional turf chemicals. Use of larvae at this late stage of development to evaluate a growth regulator such as azadirachtin may be convenient, but using this stage is not consistent with the label recommendations for application of commercially formulated neem products. These recommendations call for applications during the last two weeks of August, a time when late second and early third instars are feeding actively in turf. In the laboratory tests conducted in this chapter, I wanted to examine all the immature stages of Japanese beetles that might be treated in the field as part of my efforts to determine efficacy.

Field trials conducted by Vittum et al. (1992) that evaluated the effect of commercially formulated neem extracts on beetle grubs reduced populations 23 - 32% when compared to untreated blocks, but these population reductions were never significantly different from the control plots. Bioassays subsequently

were developed in the lab to discover whether this low level of population reduction was a result of too low a dose or of some other factor. In this chapter, resuts of laboratory assays of first instar and egg mortality treated with topical applications of azadirachtin are presented. In addition, effective concentrations of neem extracts mixed with soil were prepared and tested with first and second instars. The dosages in each case were designed to bracket proportionally either the wt./wt. or the ppm dose range reported by Ladd (1983), who had estimated an effective treatment range of 0.21- 6.8 ppm (or 0.4 to 1.6 μg of azadirachtin per insect) for late third instars based on the mean weight of 25 insects. Laboratory bioassays of all pre-pupal stages of Japanese beetles using effective concentrations of neem formulations incorporated in soil are also presented.

Materials and Methods

In order to have immature insects available for bioassay, adults were collected from Lyons, NY , and handled as described in Chapter II. Powdered azadirachtin, 95% pure, (Sigma Chemical Company) was dissolved in 95% ethanol, and 0.2 μl (range of accuracy: 10%) droplets of the test doses, diluted serially, were applied to the mid-ventral thoracic segments of insects using a Gilson-Rainin P2 pipetman with rt -10 tips. Insects were held gently with soft aluminum forceps until the solvent had dried and then placed in a 2.2 ml microcentrifuge tube.The tubes were nearly filled with a soil/peat/grass seed mixture prepared as described in Chapter II and the lids were closed. For topical application to eggs, each egg was placed at the bottom of a 2.2 ml microcentrifuge tube. A 0.2 μl drop of material was placed on each egg and allowed to dry. The microcentrifuge tubes were then filled with the soil/seeds mixture and capped as described for first instars. All insects in control

treatments received a 0.2 μl droplet of 95% ethanol similar to treatment insects. Sixty insects per treatment (with four replications) were used for the test of first instars and 50 per treatment (also with four replications) for the egg test. Microcentrifuge tubes were placed in trays and transferred to an environmental chamber, where they were maintained at 24($\pm$ 2)^{o} C on a 12:12 (L:D) h photophase.

Dose ranges were from 5 ng to 0.5 μg, diluted serially with 95% ethanol, for eggs and 0.5 ng to 0.5 μg for first instars. Twenty additional eggs and first instars per treatment were treated identically as the control insects, but placed in a separate group as 'monitors'. Monitor insects were inspected after 2 weeks and every 5 days thereafter to determine when to read the assay based on insect development. Based on observations of the 'monitors', tests were

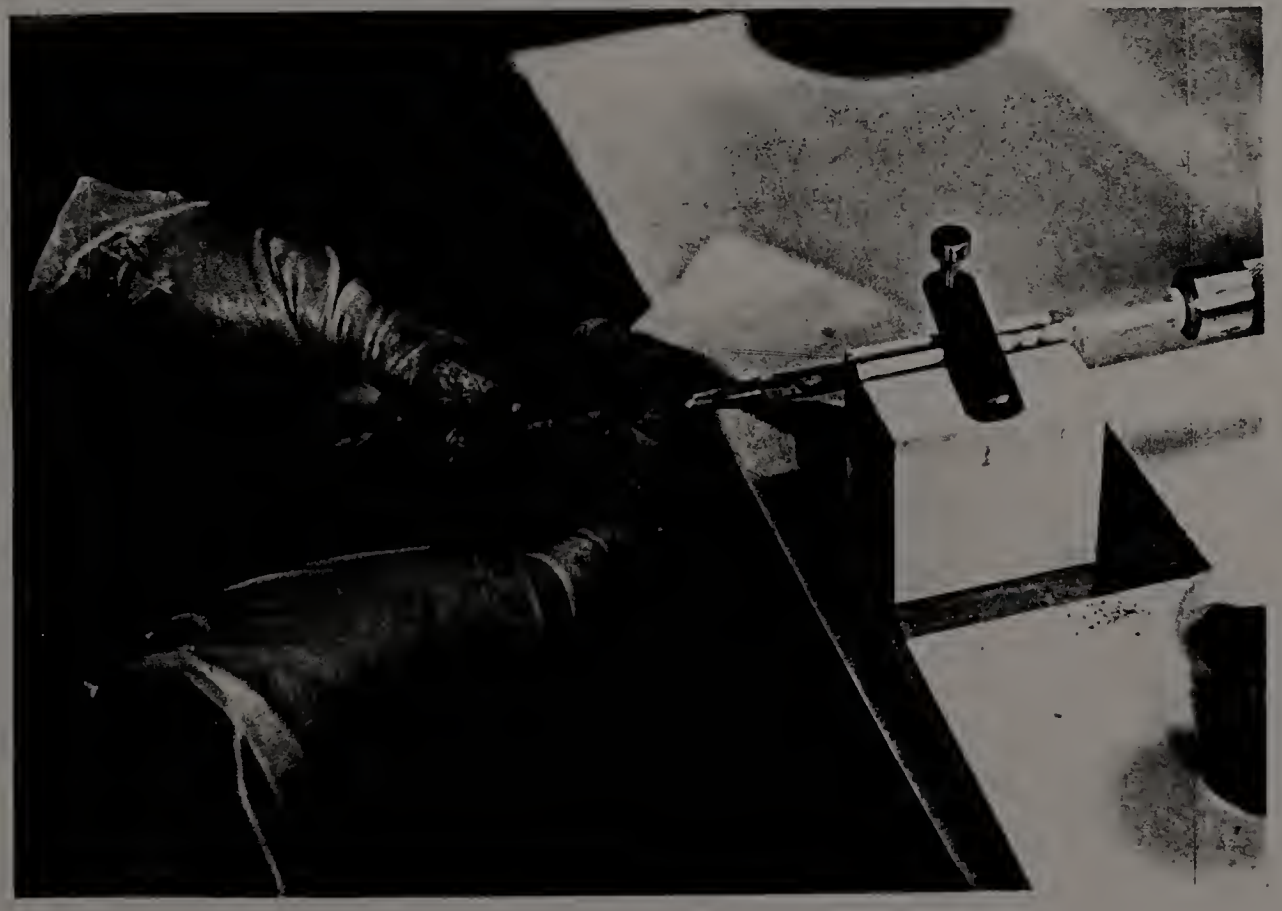


Fig. 13. Topical application of a second instar using an ISCO microinjector
K. C. Farrell photo

examined 4 weeks after treatment. Results were analyzed using Toxcalc (Tidepool Software, McKinleyville, CA). Significant differences among treatments were determined using Dunnet's Multiple Comparison (Huck et al. 1974).

Topical application of azadirachtin to second instars was done in Amherst, MA during September, 1992. Test insects were from the same Geneva-reared colony as were the eggs and first instars. Treated and untreated insects received a 1 μl droplet of solution applied to the mid-ventral thoracic segments using an ISCO microinjector and a 1 cc tuberculin syringe. The dose range was from 6 ng to 6 μg of 95% azadirachtin A. (Sigma Chemical Co., St. Louis, MO) per insect. Treatments were diluted serially with 95% ethanol. The insects were held briefly while each droplet dried and then were placed in 30 ml cups containing soil and seeds as described in Chapter II. Control insects were treated with 1 μl of 95% ethanol. Fig. 14 shows a photograph of a second instar receiving a droplet of treatment from the ISCO microinjector. Treated insects were allowed to incubate at 23 ($\pm$2)^{o} C and 75% RH on a 12:12 (L:D) h photophase in the environmental chamber for two weeks and then examined for survival. Insects were returned to the environmental chamber and examined three more times over a 150 day period. Survival data were analyzed using Toxcalc™ (Tidepool Software, McKinleyville, CA) software for generation of descriptive statistics, analysis of significant differences using Dunnet's Multiple Comparison (Huck et al. 1974), probit analysis and determination of LD_{50} values.

Results

Topical applications of azadirachtin to eggs in Geneva during August of 1992 resulted in significant mortality at the 5 μg/μl dose level (Table 7; $p < 0.05$).

Table 7. Influence of topical applications of azadirachtin on survival of eggs (N = 200 for each dose) and first instars (N = 240 for each dose) of Japanese beetles (95% pure azadirachtin in ethanol). Tests initiated in Geneva, NY, on August 6 & 7, 1992. Results sampled on August 25 and September 1, 1992.

dose (μg)	% Survival mean[a]	% Survival range	F-statistic
A. eggs			
0.0	92[a]	83 - 97	
0.005	92[a]	85 - 98	5.6312
0.05	95[a]	93 - 100	df = 4, 15
0.5	87[a]	75 - 95	crit. = 3.0556
5	75[b]	68 - 80	
B. first instars			
0.0	26	10 - 34	
0.0005	37	23 - 51	0.4903
0.005	32	24 - 48	df = 4, 15
0.05	30	28 - 35	crit. = 3.0556
0.5	30	16 - 48	

[a]Values followed by the same letter are not significantly different from the control [Dunnet's Multiple Comparison (Huck et al. 1974)]

Table 8. Influence of topical applications of azadirachtin (95% pure azadirachtin in ethanol) on survival of second instars (N = 75 for each dose) of Japanese beetles. Test initiated in Amherst, MA, 1992 on September 1 and concluded on January 29, 1993. Results analyzed at 22, 47 and 150 DAT (days after treatment).

dose (μg)	% Survival mean	% Survival range	F - statistic
A. 22 DAT			
0.0	92 N.S.	88 - 100	
0.006	85	80 - 92	0.625
0.06	95	92 - 100	df = 4,10
0.6	91	84 - 100	crit. = 3.478
6	91	80 - 100	
B. 47 DAT			
0.0	84 N.S	80 - 80	
0.006	71	48 - 88	1.628
0.06	92	84 - 100	df = 4,10
0.6	77	68 - 88	crit. = 3.478
6	88	80 - 96	
C. 150 DAT			
0.0	81 N.S.	76 - 84	
0.006	59	36 - 84	1.827
0.06	85	80 - 96	df = 4,10
0.6	69	60 - 76	crit. = 3.478
6	77	68 - 92	

N.S. - no significant differences (Dunnett's Multiple Comparison, $P<0.05$)

Estimates of the LD_{50} (dose at which 50% of the population is killed) using probit analysis are not reported, as the Chi-Square statistic for the goodness-of-fit of the data was well below the critical statistic (Chi-square statistic = 2.94; critical = 9.21) and a probit line was not calculated. Mortality was high in all treatments of first instars, as shown by the per cent survival means (Table 7), and the analyses of the survival outcomes produced no differences among treatments. As a consequence, LD_{50} estimates for this assay were not calculated. These tests were ended after the first sampling period (August 25 - September 1). The tests were ended at that time because it was a protocol for most assays conducted in the lab in Geneva to end them after the first examination, and I had not realized at that point that interesting information about treatments of insects with azadirachtin could be obtained by holding tests longer and looking at the survival of larvae on several sample dates. Results of the bioassay of second instars are shown in Table 8. Data were analyzed for the three readings (DAT, or days after treatment) shown in Table 8. Survival data among the treatments are very similar, and no significant differences among treatments were found ($P<0.05$). As survival only fell below 50% for one treatment at 150 DAT, no LD_{50} estimate was made.

Discussion

We hoped to understand the impact of azadirachtin, on field populations of Japanese beetles by treating egg and larval stages in the laboratory. However, first instars were highly sensitive to handling or possibly some other condition of the bioassay, as was seen by the very low survival observed in all treatments (Table 7). The high mortality of first instars may have also been due to the size of the 2.2 ml containers in which they were stored. A smell of alcohol was detectable upon opening many of the microcentrifuge tubes, and it is possible the ethanol used to dissolve the azadirachtin volatilized during the

time first instars were stored in the microcentrifuge tubes, ultimately killing them. As a result of these outcomes, first instars appeared to be very poor candidates for topical application assays.

Neither the results of applications to eggs nor to second instars provided information for the estimation of LD_{50} values. While significant differences among treatments were found in the assay of eggs, a wider dose range or longer trial period in an assay of eggs would presumably be needed to obtain the range of mean survivals needed for probit analysis to be possible. Survival among the second instar treatments was too high for probit analysis to be performed, and tests of these insects would probably require a higher dose range for differences among mortality outcomes to become apparent.

Another problem with the use of the topical application method was that the effects of neem extracts related to its activity as a toxin of the midgut were not measured, and presumably the mortalities which occurred were due to other aspects of azadirachtin toxicity. Insect mortality due to combined modes of action interacting to produce the lethal effects of azadirachtin has been reported by several authors (Ascher 1993, Mordue & Blackwell 1993). From the preliminary test of second instars, it did seem that some toxic effects occurred at pupal eclosion, when a number insects were not able to free themselves of the larval exuvium. The results of my topical application tests suggested that assays needed to be run for longer periods so that these different causes of insect mortality could be measured.

I concluded at the end of 1992 that a better model for the design of bioassays utilizing azadirachtin included holding all tests until all possible insects had emerged as adults. It would have been possible to repeat topical application tests in 1993, using the new sampling criteria and attempting to control more for mortality due to handling. Although repeating these tests was

considered, it was decided that additional tests (and especially topical applications to first instars) would be unlikely to provide a reliable estimate of beetle sensitivity to azadirachtin. Therefore, I concluded that another assay method needed to be developed in order to accomplish the goal of testing all immature stages.

Bioassays Using the Effective Concentration Method

Introduction

After my first year of observations of Japanese beetle grubs, I developed a hypothesis that under conditions of grub management in the field, an application of azadirachtin-based insecticide seemed to be as likely to affect grub survival via ingestion of the active ingredient as it was to kill them by crossing the cuticle. To test this hypothesis, I developed a new assay that evaluated actual turf formulations of neem extract mixed with the soil in which the treated insects lived, and avoided the use of additional water or alcohol in preparation of the treatments. Instead of estimating an LD_{50} based on mean weight values of a sample of grubs, the new test would estimate the effective concentration of active ingredient in the soil, or EC_{50} (Matsumura, 1975), at which 50% of the tested grubs would perish.

One possible advantage of this method was that it might lessen the mortality of first instars because they would not be handled as much as they were during topical application. Eggs could be placed in cups of treated soil and the resulting neonates allowed to develop undisturbed for the first month, a time when they seemed most sensitive to handling. It was also hoped that this method would provide for a more successful evaluation of the other immature stages. In the second year of the study, beetles were also maintained until adult

emergence or death in order to assess the impact of neem extracts on adult emergence.

Materials and Methods

The concentration of azadirachtin, the principal active ingredient of the commercial formulations, was the basis for the calculations for assay doses in each replication. The soil proportions required for each dose were weighed directly into a tared 3.8 liter glass jar on a Mettler 2000 scale. The remaining weight of formulation was then pipetted onto the soil until the total weight, either a 500 g or 800 g amount (depending on the number of insects used in the replication) was reached. Three ceramic tumblers were added to the jar and it was capped. Each mixture, as well as untreated soil controls were rolled on a Norton rotary mixer for 30 minutes. All treatments were transferred to clean plastic bags and allowed to sit unsealed for 2 or 3 h to allow the alcohol present in the formulation to volatilize. Grass seed was then added to each bag of soil and the contents of the bag were mixed thoroughly.

Small cups (30 ml) were filled to 80% with the appropriate soil mix, a grub or egg was placed in the center of the soil mix, and the cup was capped. Field collected insects were allowed 10 minutes to burrow into the medium. Those failing to do so during that time were replaced with another insect. Test insects were placed in treatment groups by use of a random number table. The cups were placed on trays and enclosed (25 or 30 cups per bag) in clear plastic bags. Each test included 25 (second or third instars) or 30 (eggs or first instars) insects per replication. All treatments were replicated at least three times. Beetles were kept in a Percival environmental chamber at 23^{o} C and 70% RH with a 12:12 (L:D) h photophase before and after treatment.

Ranges of treatment for all immature stages are shown in Table 9. Bioneem™, a product of Ringer Corp., was used in assays during 1992, and Margosan-O™, a product of W. R. Grace & Co. was used during 1993 and 1994. Application rate ranges were from 0.05 to 50 ppm in 1992. This range was decreased in 1993, because the grass seed did not germinate in treatments above 10 ppm, and it was found that significant differences in grub survival were measurable at 10 ppm. Autumn tests were placed in environmental chambers for two weeks and then examined weekly until the test was ended during the subsequent winter. In 1992, first instar tests were observed for 27 days only, and tests of second and third instars lasted only until the onset of pupation. During 1993, assays were examined until all but the last few individuals reached the adult stage. A preliminary study (Roy, unpublished data) had shown that third instars which failed to pupate more than two weeks after the peak of pupation would eventually perish after a long period as a larva without ever pupating. Spring tests were inspected every 5 to 7 days until two weeks after the peak of adult emergence. Test results (calculated as insect survival on selected days after treatment) were analyzed using Toxcalc software, as in previous trials, for provision of dose-response plots, determination of significant differences among treatments, and estimates of EC_{50}'s for each assay.

Results

Because of the very long time required for mortality patterns to appear in the tests, LT_{50} estimates were not calculated. Instead, the first reading at which larval survival fell below 50% of the initial population in each treatment is reported in Table 9. The results of EC_{50} estimates for each bioassay are shown in Table 10. The appropriate hypothesis tests used to

Table 9. Lethal time estimates in bioassays of neem formulations on Japanese beetles. Estimate shown as the first date (DAT, or day after treatment) in a treatment when % survival fell below 50%. Bioassays of larvae in 1992 with Bioneem™ as effective concentrations in soil, and of larvae and eggs in 1993 with Margosan - O™ as effective concentrations in soil.

A. Eggs

1993 (N = 90/treatment)

dose (ppm)	DAT
0.0	57
0.05	53
0.1	63
1	26
5	26
10	26

B. First instars

1992 (N =125/treatment)

dose (ppm)	DAT
0.0	27
0.05	27
0.5	27
5	27
50	8

1993 (N = 30/treatment)

dose (ppm)	DAT
0.0	27
0.05	45
0.1	45
1	27
5	27
10	27

C. Second instars

1992 (N = 75/treatment)

dose (ppm)	DAT
0.0	151
0.05	151
0.5	151
5	43
50	17

1993 (N = 75/treatment)

dose (ppm)	DAT
0.0	65
0.05	71
0.1	85
1	68
5	32
10	27

continued to p. 52

Table 9 continued

D. Third instars, autumn

1992 (N = 75/treatment)

dose (ppm)	DAT
0.0	151
0.05	151
0.5	151
5	43
50	17

1993 (N = 100/treatment)

dose (ppm)	DAT
0.0	65
0.05	71
0.1	85
1	68
5	32
10	27

E. Third instars, spring

1993 (N = 75/treatment)

dose (ppm)	DAT
0.0	32
0.05	32
5	47
7	47
8.5	24
10	24

1994 (N = 100/treatment)

dose (ppm)	DAT
0.0	52
0.1	52
1	40

Table 10. Effective concentration estimates (EC_{50}) in bioassays of neem formulations on Japanese beetles . Dates (DAT, or day after treatment) in a treatment when the sampled responses of insects (survival) to the neem formulations were significantly different among treatments. Bioassays of larvae in 1992 with Bioneem™ effective concentrations in soil, and of larvae and eggs in 1993 with Margosan - O™ effective concentrations in soil.

A. Eggs

DAT	significant doses (ppm)	EC_{50}(ppm)[1]	95% confidence limits
1993	(N = 90/treatment)		
28	1, 5, 10	0.82	0.61 - 1.10

B. First instars

DAT	significant doses (ppm)	EC_{50}(ppm)	95% confidence limits
1992	(N =125/treatment)		
15	50	24.52	19.85 - 30.27
27	50	7.76	4.72 - 12.76
1993	(N = 30/treatment)		
45	5,10	not estimated	trim > 50 %

C. Second instars

DAT	significant doses (ppm)	EC_{50}(ppm)	95% confidence limits
1992	(N = 75/treatment)		
43	5, 50	5.16	3.78 - 7.03
53	5, 50	1.51	1.40 - 1.63
70	5, 50	1.44	1.31 - 1.59
1993	(N = 75/treatment)		
	none	not estimated	
56	10	3.55	2.88 - 4.36

continued to p. 54

Table 10 continued

D. Third instars, autumn

DAT	significant doses (ppm)	EC_{50}(ppm)[a]	95% confidence limits
1992	(N = 75/treatment)		
27	10, 20	13.40	10.6 - 16.92
35	10, 20	10.41	9.28 - 11.68
66	5, 10, 20	5.15	4.33 - 6.12
1993	(N = 100/treatment)		
30	none	not estimated	
50	10	3.43	2.82 - 4.16
72	10	1.61	1.33 - 1.95

E. Third instars, spring

DAT	significant doses (ppm)	EC_{50}(ppm)	95% confidence limits
1993	(N = 75/treatment)		
30	10	7.40	7.21 - 7.61
45	8.5, 10	7.20	6.69 - 7.76
1994	(N = 100/treatment)		
28	none	not estimated	
42	none	not estimated	
52	1	0.48	0.37 - 0.64

[a]Point estimates of EC_{50} (concentration at which 50% of treated insects are killed) performed using the Spearman-Karber trimmed method of estimation

Table 11. Pattern of adult emergence from bioassays of neem extracts on the survival of Japanese beetles, 1992 - 1994. Tests initiated at the life stages shown and followed until the peak of adult emergence (1992) or until all possible adults had emerged (1993). Treatments shown as parts per million (ppm) of azadirachtin, and the total number of emerged adults is shown below the line under each treatment.

A. Eggs

1993, N = 90/treatment

solvent control	water control	0.05	0.1	1	5	10
3	3	1	1	0	0	0

B. First instars

1993, N=30/treatment

solvent control	water control	0.05	0.1	1	5	10
1	0	3	2	0	0	0

C. Second instars

1992, N=75/treatment

water control	0.05	0.5	5	50
9	14	6	0	0

1993, N=75/treatment

water control	0.05	0.1	1	5	10
1	2	1	1	0	0

D. Third instars, autumn

1992, N=75/treatment

water control	1.25	2.5	5	10	20
21	10	1	0	0	0

1993, N=100/treatment

water control	0.05	0.1	1	5	10
0	0	1	0	0	0

E. Third instars, spring

1993, N=75/treatment

water control	0.05	5	7	8.5	10
37	35	2	1	0	0

1994, N=100/treatment

water control	0.1	1
41	47	12

test for significant doses among treatments were selected in each assay by a flow chart embedded in the software (Toxcalc). The level of precision for all hypothesis tests was set at the $p<0.05$ alpha level. The results, recorded weekly as survival per treatment, were related to the outcomes of the previous week. Because of this, the assumption of independence for each set of results was violated, and a parametric analysis of the treatments, as is the case when using logit or probit analysis, was not appropriate. Instead, point estimates for the EC_{50} values for all assays were made using a non-parametric method; the trimmed Spearman-Karber format reported in Table 10. Whenever the trim of the data evaluated was 50% or more, no EC_{50} value was calculated.

Where possible, adult emergence patterns were also reported. A summary of these patterns appears in Table 11. Adult emergence was different from year to year, presumably based in part on the vigor of the population in the year of each assay. For example, adult emergence from insects reared in 1993, when very little rain fell in June and July, was particularly low, as was larval survival. The egg hatch from adults collected in 1993 to begin a laboratory colony to use in bioassays was also lower than the average annual hatch of 90% reported by Fleming (1972). The time at which mortality occurred was also different between 1992 and 1993. In 1993, almost all mortality occurred at the larval stage, but in 1992, mortality was seen as well at the larva-pupa molt. Insects dying at this time were not able to exit the split larval skin, or slip their old head capsules (Fig. 14). Occasionally, insects were unable to form a pupa, creating an intermediate morph containing both larval and pupal characters. In some cases, a misshapen adult emerged with deformed wings (Fig. 15), and died soon after eclosion. These deformed adults were recorded as mortalities.

Survival in all treatments in the assay of eggs in 1993 is shown in Fig. 16. At 28 DAT, significant differences using the Bonferonni Adjusted T-test were found at 1, 5 and 10 ppm (Table 10). The EC_{50} estimate for that date was 0.82 ppm. A dose response plot of these results appears in Fig. 17. Median survival among treatments at that date was 35%.

Survival of first instars among treatments in the assay prepared in1992 is shown in Fig. 18. Significant differences among treatments were detected at 15 and 27 DAT using Steel's Many-One Rank test, and EC_{50} estimates of 24.5 and 7.8 ppm on those two dates are shown in Table 10. Dose response plots of these data are shown in Fig. 19. Median survival was not reported on the plots, an indication that the wide distribution of the data did not provide a range for a reliable EC_{50} estimate. A better estimate of the EC_{50} for the test would have been more likely had the tests been kept for a longer period of time. No adult survival data were available for this test because of premature termination of the study.

Larval survival in the assay of first instars in 1993 was less than 10% in all but one control group. Four replications were prepared of this assay, but survival after the second week post-treatment was so low that data were collected after that for only one replication. The survival outcomes among treatments in this test are shown in Figure 20. Fisher's exact test was used to test for differences in this assay, and significant differences at 45 DAT (Table 10) were detected at the 5 and 10 ppm level ($p=0.0002$). A Spearman-Karber estimate of the EC_{50} value was not performed, as the trim of data needed to estimate it was greater than 50%. Dose-response plots of the results were also not possible as they were based on this estimate. Adult emergence for this test, as shown in Table 11, never exceeded three insects.

Survival among treatments in the 1992 assay of second instars is shown in Fig. 21. Dunnett's Multiple Comparison was selected to assess significant differences among the treatments; differences which were found at 43, 53 and 70 DAT for the 5 and 50 ppm treatment groups (Table 10). The EC_{50} estimates on these dates were 5.16, 1.51 and 1.44 ppm respectively. In Fig. 22, dose-response plots of the survivorship at 43 and 70 DAT display a similar pattern to one another, with median survival shown as 51.5 and 50.2% on the two dates. The dose-response plot at 53 DAT, not shown in Fig. 22, was virtually the same as those plotted at 43 and 70 DAT.

The survival among treatments of second instars assayed in 1993 is shown in Figure 23. Only one EC_{50} estimate was made, at 56 DAT, when significant differences were found at 10 ppm using Dunnett's Multiple Comparison (Table 10). The EC_{50} estimate was 3.55 ppm. Dose-response plots of the 1993 assay of second instars for 14 and 56 DAT are shown in Fig. 24. The median survival line (24%) at 56 DAT is above the mean value for survival at 5 ppm, suggesting that 5 ppm might also be a significant dose. The t-value of 5 ppm (Dunnet's Multiple Comparison) at 56 DAT was 2.25, with a critical 't' of 2.29, implying that this mean value, while nearly significant, represented a very wide range of outcomes, as shown by the minimum-maximum range of these data. As a result, the confidence limits, set by the standard error for the values represented in the critical 't', did not include this dose as one significantly different from the control t-statistic.

Fig. 25 shows survival in treatments of the assay of third instars conducted in 1992. At 27 and 35 DAT, significant differences were found at the 10 and 20 ppm using Dunnet's Multiple Comparison. At 66 days, significant differences were also found at 5 ppm. The EC_{50} estimates for these three dates were 13.4, 10.4 and 5.15 ppm (Table 10). A comparison of the dose-response

plots for survival on these dates (Fig. 26) shows the plots at 27 and 35 DAT are quite similar, with median survivals of 58% and 43.2%. At 66 DAT, the median survival among treatment groups drops to 22.1%. The mean survival value for treatments 5 and 2.5 ppm falls below this line, suggesting that survival in these treatments might be different from that of the control groups. The range of the values at 2.5 ppm is too wide, however, for survival at this dose to be considered significantly different from survival in the control groups.

Survival among treatments of third instars collected in the field in the late summer and used for assays in 1993 is shown in Fig. 27. Evaluations of treatment outcomes were made at 30, 50 and 72 DAT. As the trim required for the Spearman-Karber estimate was greater than 50% at 30 DAT, no EC_{50} was made for that date. EC_{50} values at 50 and 72 DAT were 3.43 and 1.61 ppm with significant differences found at 10 ppm (Table 10). The dose-response plots for this test (Fig. 28) show a very wide range of the data in all three graphs, and a low median survival of 31.8% at 30 DAT, 15.7% at 66 DAT and 8.9% at 72 DAT. Median survival with such a low value, and representing such a wide range of outcomes suggests that the assay of third instars in 1993 produced particularly unreliable results for the estimation of EC_{50} values.

The results of the assay of third instars (harvested in the spring of 1993 from South Hadley, MA) are shown in Fig. 29. The test of significance using Dunnett's Multiple Comparison on these treatments (Table 10) showed that survival in the 10 ppm group was significantly different at 30 DAT and both 8.5 ppm and 10 ppm were at 45 DAT. The EC_{50} estimates on these two dates were 7.4 and 7.2 ppm. Dose-response plots of survival at these two dates are shown in Fig. 30.

The bioassay conducted in 1994 with insects collected in Braintree, MA (Fig. 31) was designed to examine the sub-lethal effects of low doses of Margosan-O on emerged adults, and consequently has a narrower dose range. Because of this, estimates of significant differences among treatments or of EC_{50} values were not possible until 52 DAT, when significant differences were found at 1 ppm using Dunnet's Multiple Comparison. The EC_{50} estimate for that date was 0.48 ppm (Table 10). A dose response plot of the data at that time (Fig. 32) showed a median survival of 41.6% and not very much spread of the data. It is possible to infer from these data that even though the EC_{50} estimate had a low value, it appeared to be a precise estimate.

Discussion

The use of the effective concentration method of bioassay provided a means for deriving estimates of acute doses of neem extracts on immature Japanese beetles in 1992 and 1993 tests. Differences in the responses of the same instar from one year to the next remain unexplained, although vigor in the populations used may have played a role in the outcomes observed. In particular, eggs or neonates which were more stressed at the onset of the test may have been more vulnerable to sub-lethal effects of azadirachtin. First instars continued to be the most difficult of the immatures to investigate, but the results from the 1992 test of first instars seem to indicate that assay of first instars is possible when a healthy population of insects is available.

These studies also yielded information regarding the optimal timing of sampling. In most cases, data seemed easiest to compare at 4 to 8 weeks after treatments of second and third instars were prepared in the fall, or at 4 to 6 weeks in the spring. Reasonable estimates of treatment of eggs or first instars could be made one month posttreatment. Although insect development in

environmental chambers tended to be slower than occurs in field populations of Japanese beetles, we can infer from the estimated lethal times shown in the laboratory tests (Table 9) that field trials of these insects should usually be assayed later than 30 days posttreatment for results to be reliable.

Mortality of immatures seemed to continue throughout the tests performed in this study. In 1992, in particular, mortality appeared to be the result of a combination of events, as insects perished as larvae, at the molt of larvae to pupae or at adult eclosion. It would be advisable in future assays of Japanese beetles to partition the clearly acute mortalities at found doses above 1 ppm from those sub-acute (or chronic mortalities) observed below that treatment level. As azadiractin appears to act as a stomach poison at the higher doses and to influence the beetles as a growth regulator at the lower doses, the separation of dosage levels might aid in development of EC_{50} estimates based solely on acute mortalities.

As is frequently observed in the dose-response plots for bioassays, survival of insects in treatments from 0.05 to 0.1 ppm was often better than in control populations. A similar occurrence has been reported in tests of *Triboleum castaneum* Herbst by Ramachandran et al. (1988) and Mukherjee & Ramachandran (1989). These authors attributed the results to a 'hormetic effect' stemming from the structure of the azadirachtin molecule, but it was also possible that the anti-microbial activities of azadiractin operating at these lower doses was improving the relative health of insects in these treatments by killing some of the microbial inhabitants of their mid or hindguts. An analysis of the reproductive organs of adults emerged from treatment groups at these dose levels would reveal whether the emerged adults were as reproductively viable as those that emerged in the control groups.

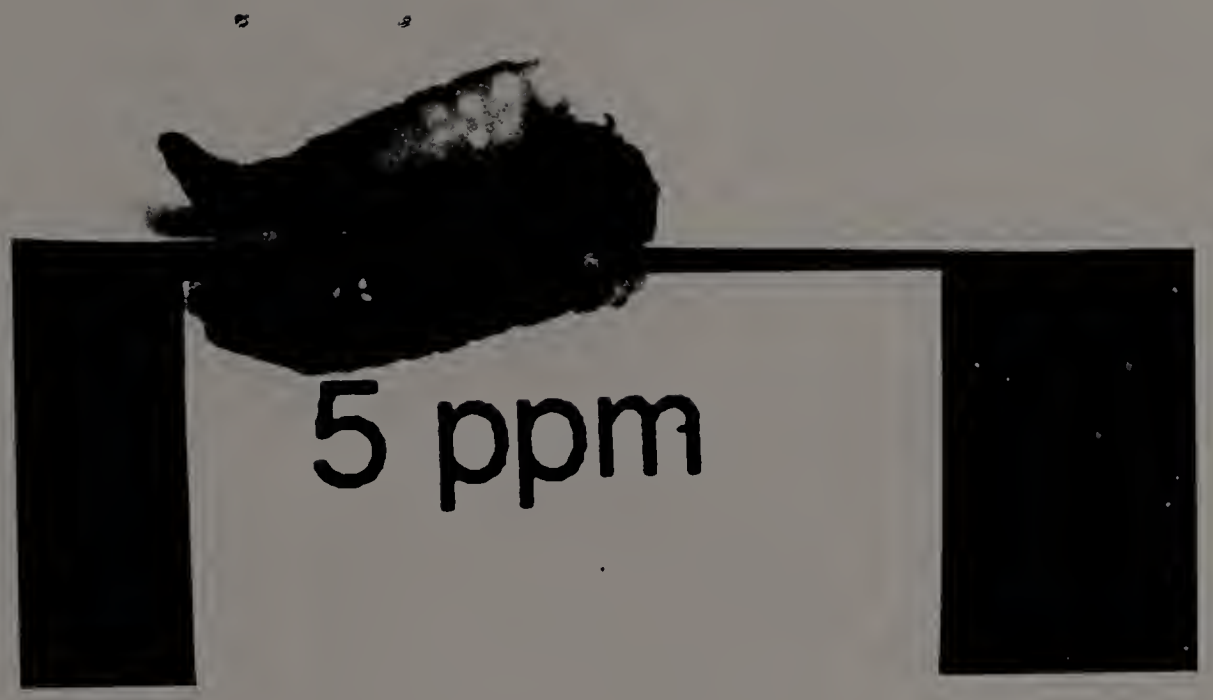


Fig.14. Deformation of a larva at the pupal molt.
P. J. Vittum photo

Fig. 15. Deformation of pupae at the adult molt.
P. J. Vittum photo

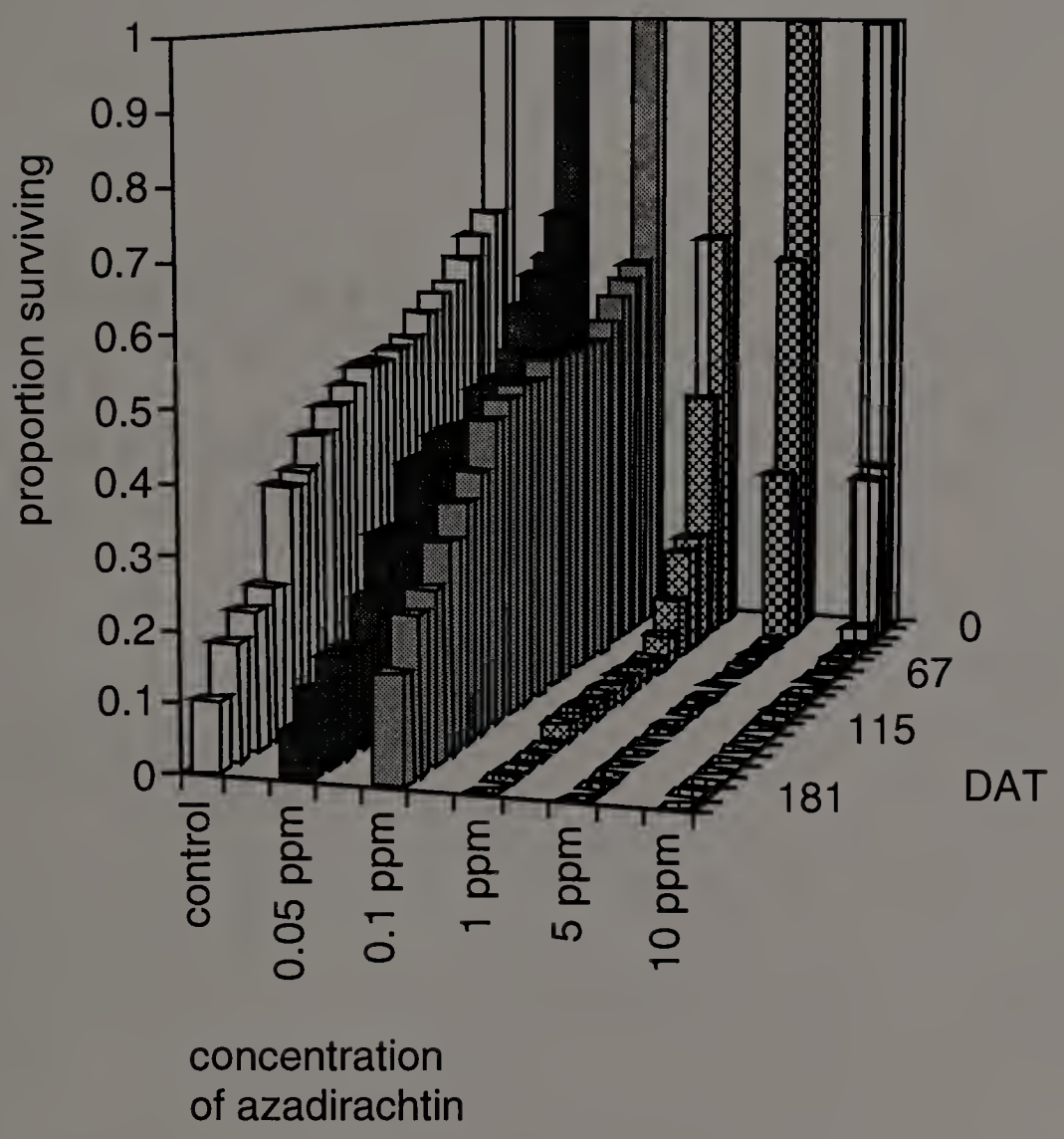
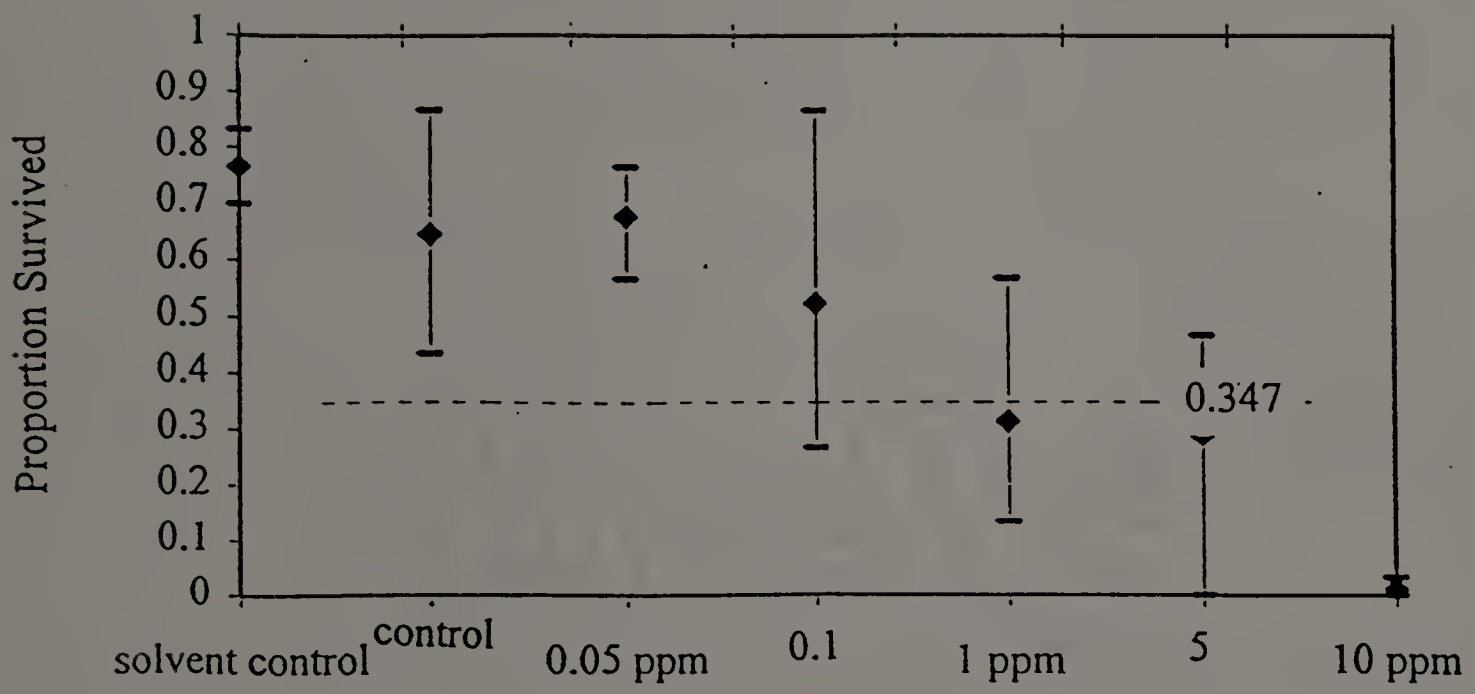


Fig. 16. Survival of Japanese beetle eggs treated with Margosan-O™ and examined weekly beginning 28 days after treatment (DAT). Test initiated August 13, 1993 and concluded on January 5, 1994.



Concentration - (ppm)
28 DAT

Fig. 17. Dose response plot of the survival of Japanese beetle eggs 28 days after treatment (DAT) with Margosan-O™. Test initiated August 31, 1993. Median survival among treatments (35%) shown as a dashed line in the center of the plot. Mean survival in treatments shown below this line are significantly different ($p<0.05$) from the mean survival of control groups.

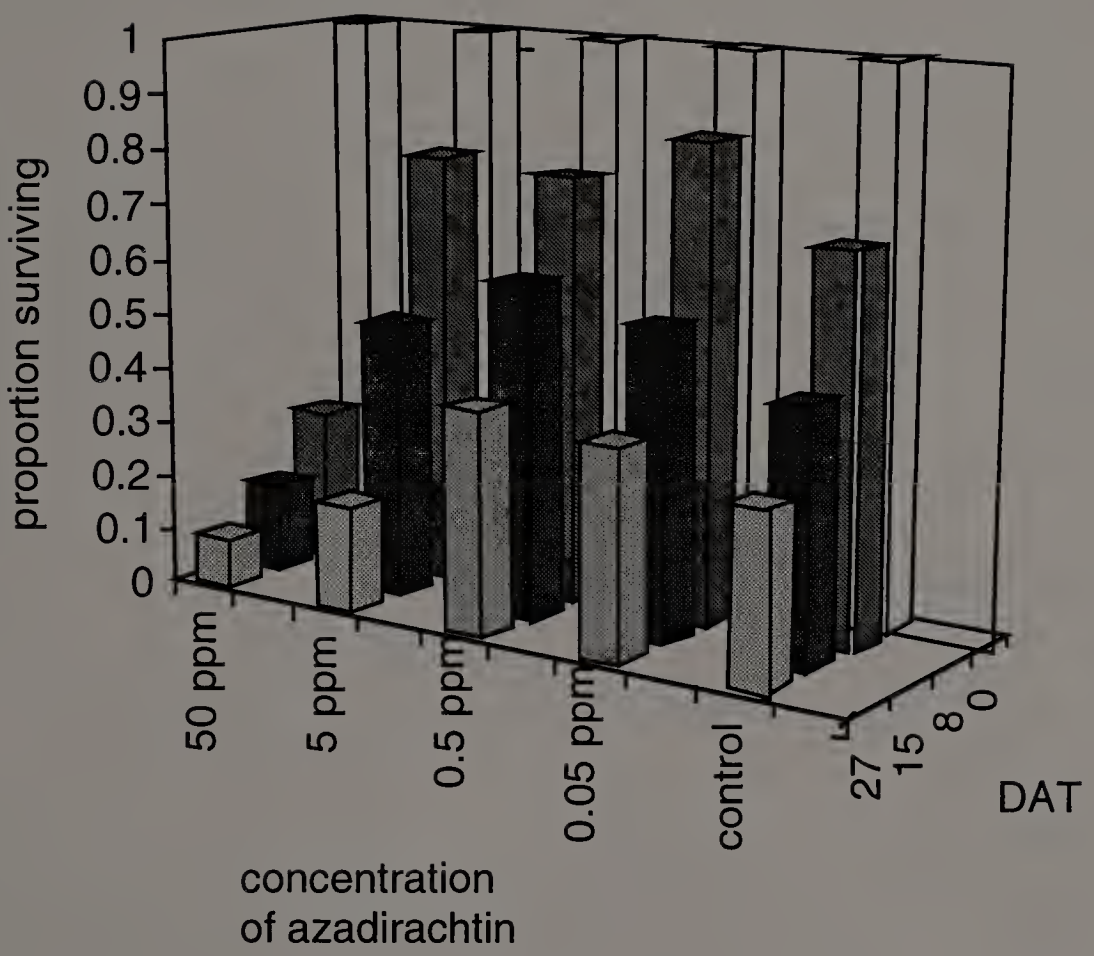
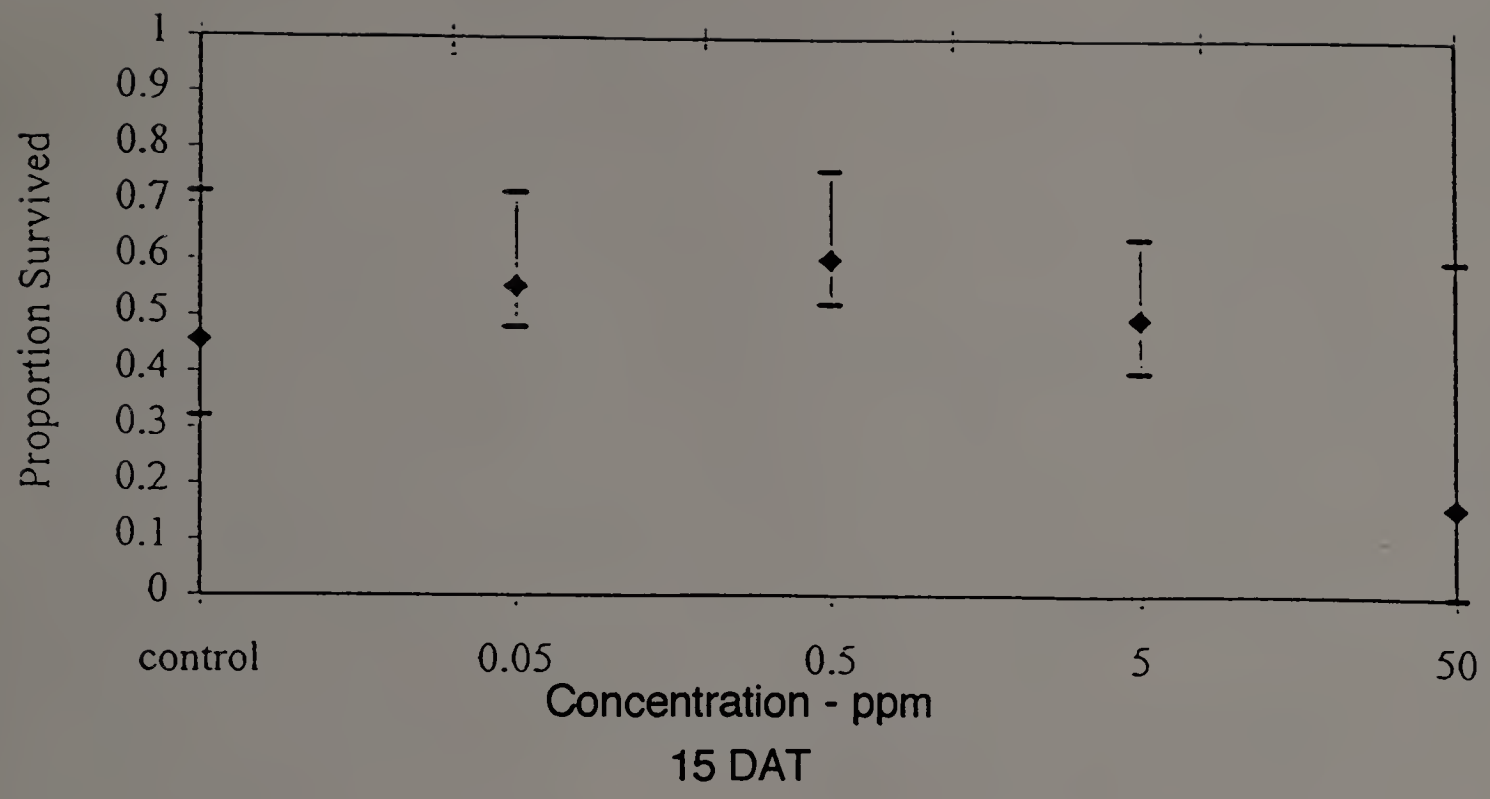


Fig. 18. Survival of Japanese beetle first instars treated with Bioneem™ and examined weekly beginning 15 days after treatment (DAT). Test initiated August 26, 1992 and concluded September 22, 1992.



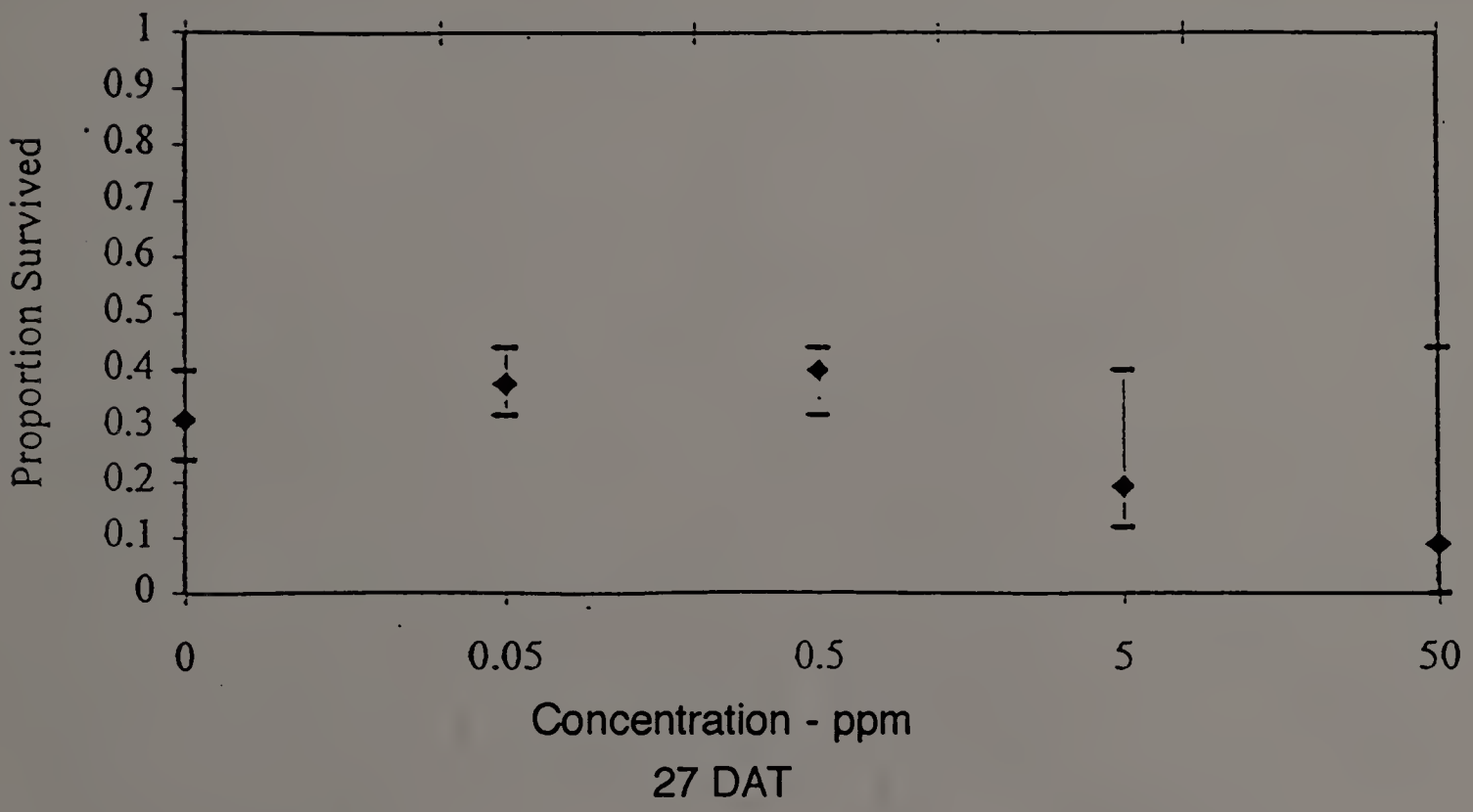


Fig. 19. Dose response plot of the survival of Japanese beetle first instars treated with Bioneem™ and examined at 15 and 27 days after treatment (DAT). Test initiated August 26, 1992 and concluded September 22, 1992. No significant differences ($p<0.05$) among treatment were seen on either date

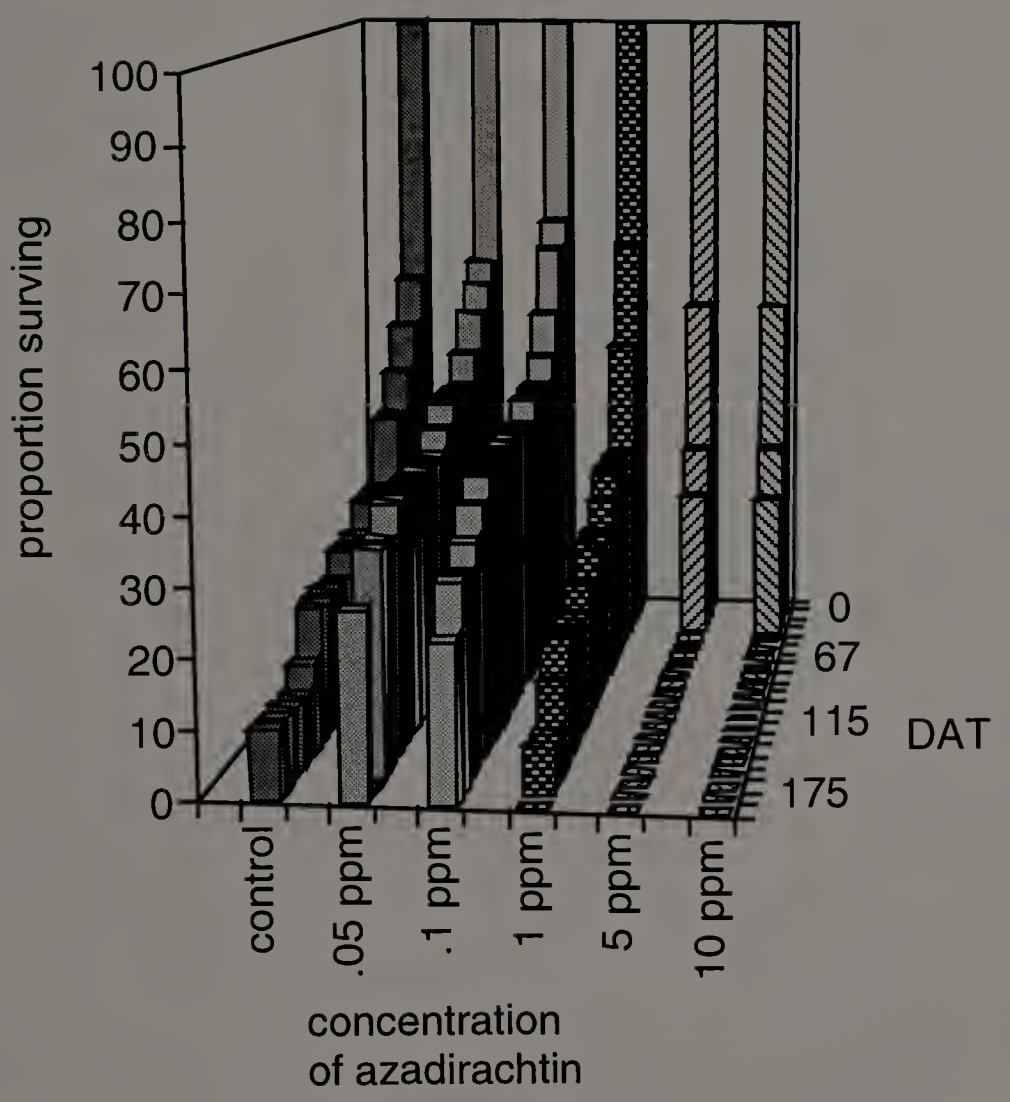


Fig. 20. Survival of Japanese beetle first instars treated with Margosan-O™ and examined weekly beginning 30 days after treatment (DAT). Test initiated August 13, 1993 and concluded January 12, 1994.

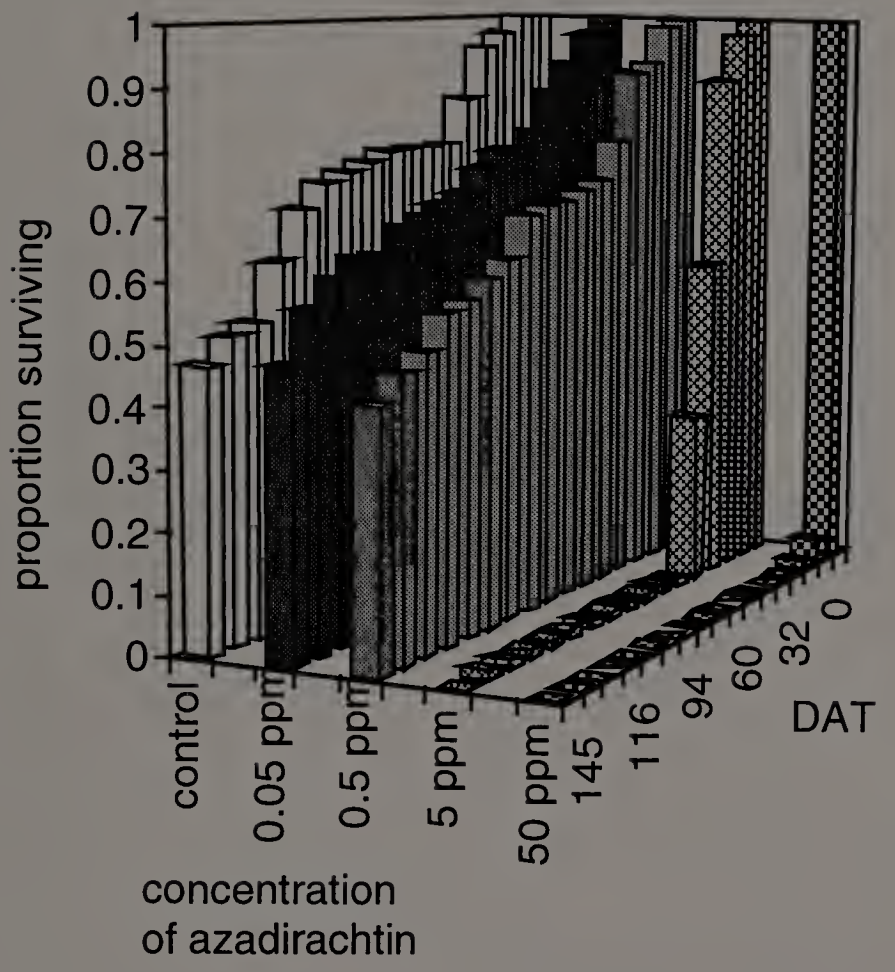
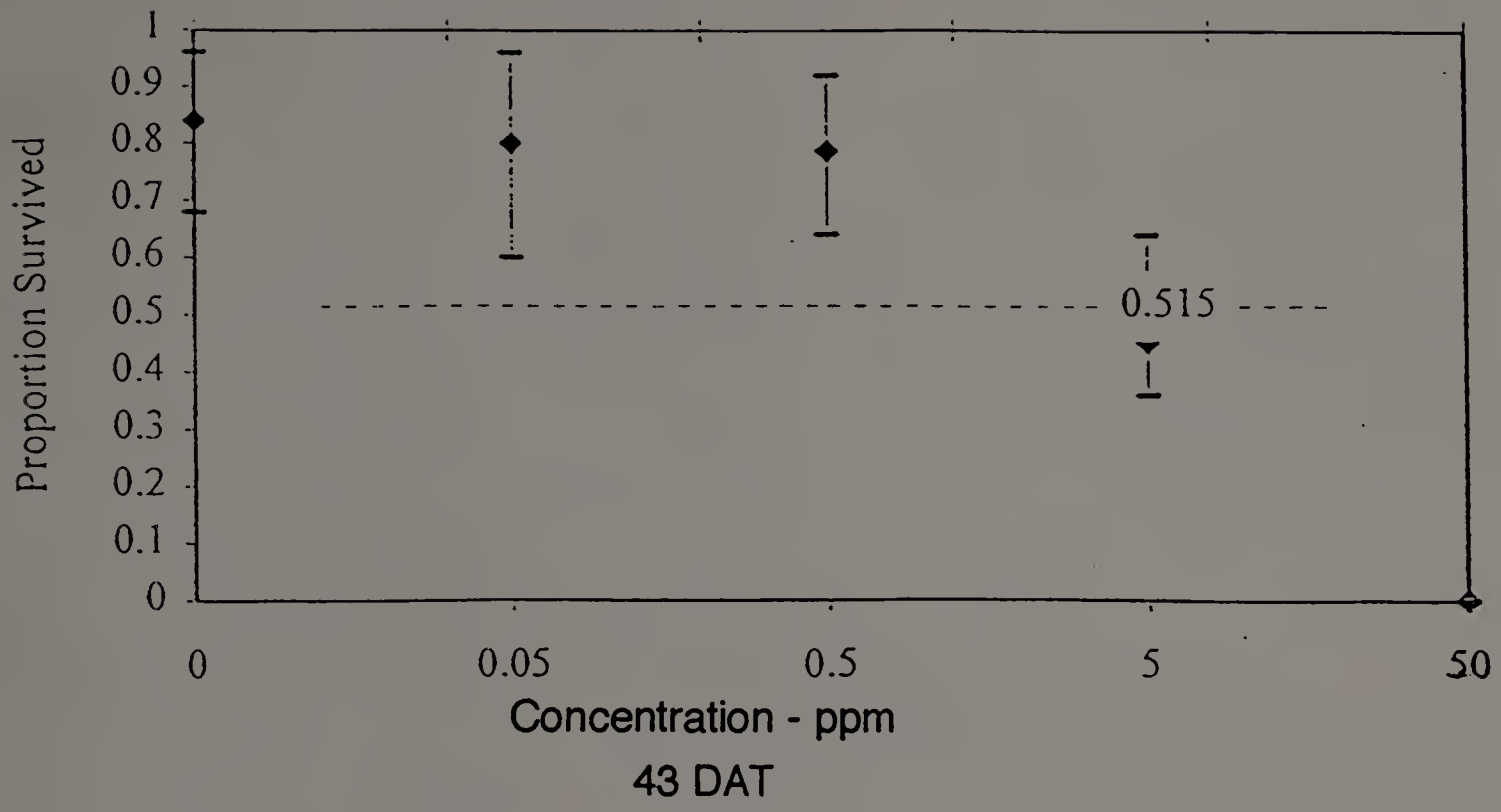


Fig. 21. Survival of Japanese beetle second instars treated with Bioneem™ and examined weekly beginning 32 days after treatment (DAT). Test initiated August 31, 1992 and concluded December 4, 1992



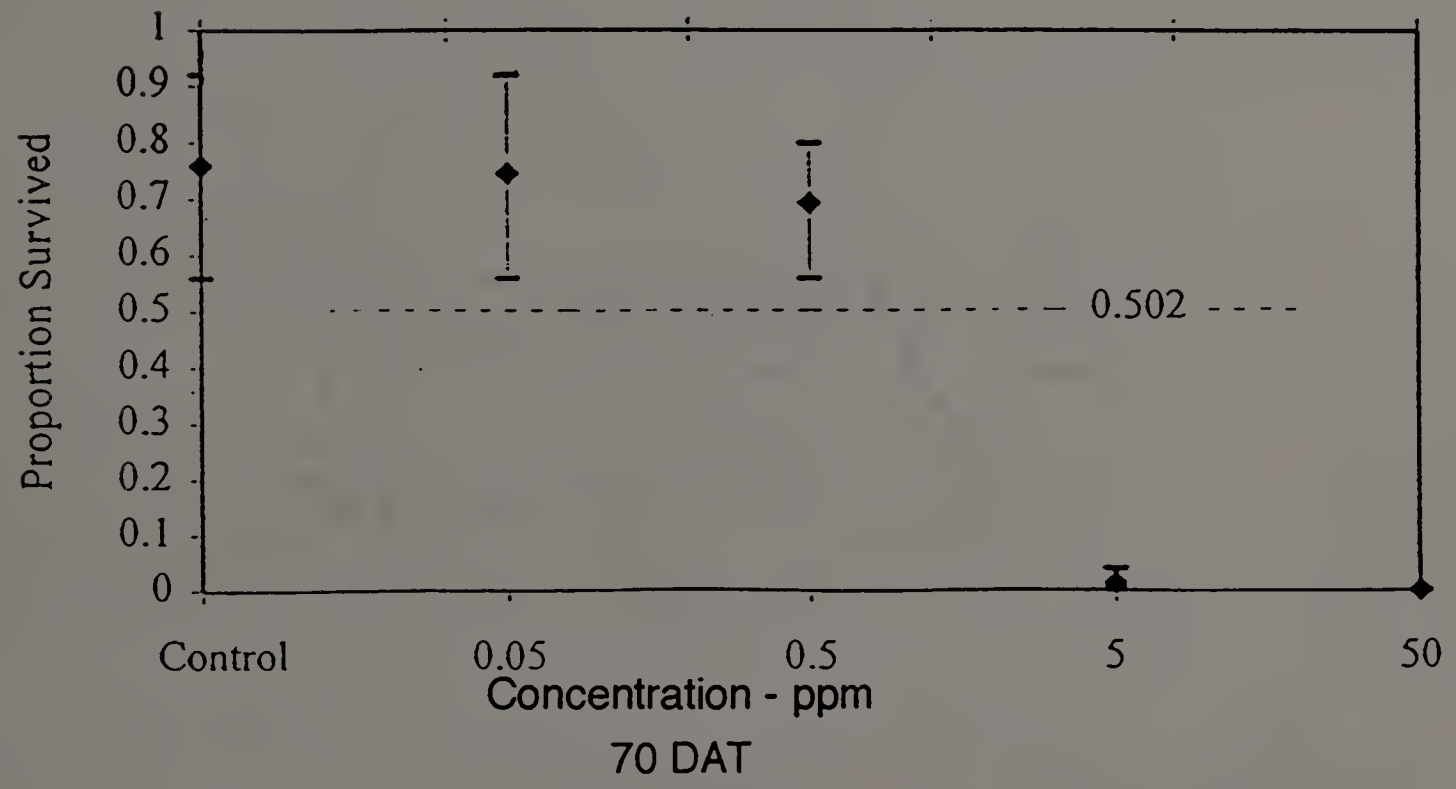


Fig. 22. Dose response plots of the mean survival of Japanese beetle second instars treated with Bioneem™ and sampled at 43 and 70 days after treatment (DAT). Test initiated September 1, 1992 and concluded December 4, 1992. Median survival among treatments (52 and 50%) shown as a dashed line in the center of each plot. Mean survival in treatments shown below this line is significantly different ($p<0.05$) from the mean survival in the control group.

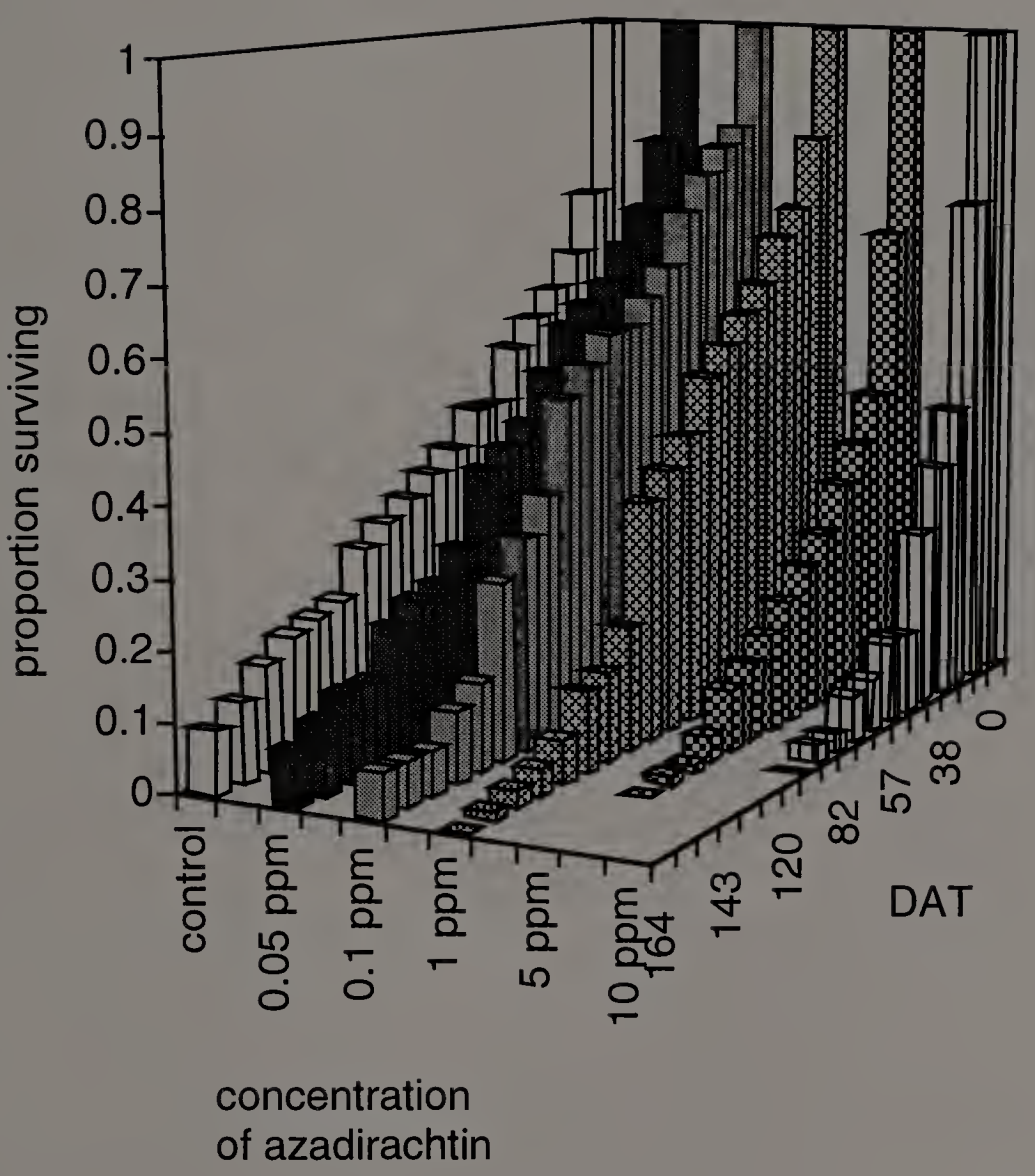
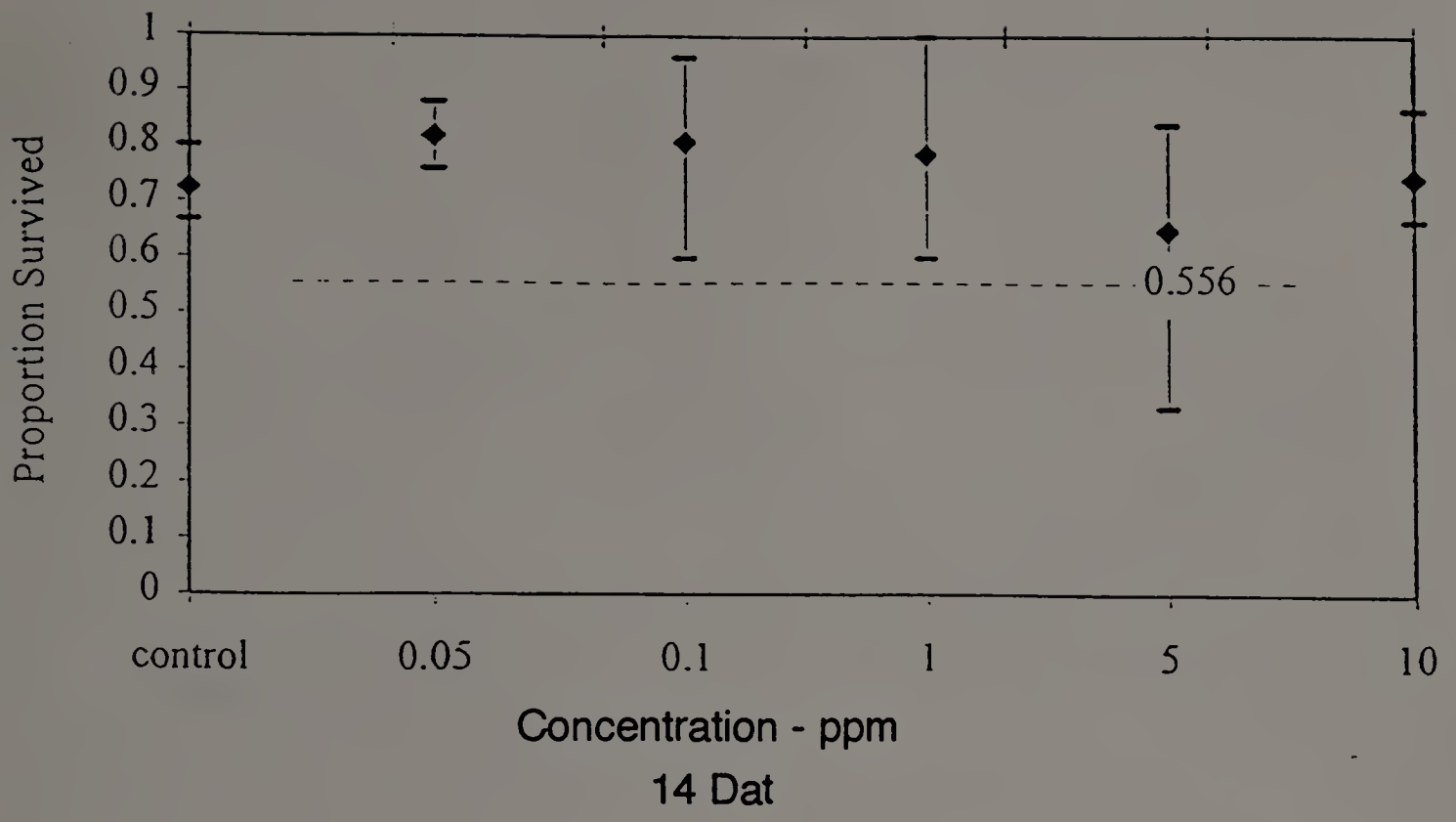


Fig. 23. Survival of Japanese beetle second instars treated with Margosan-O™ and examined weekly beginning 14 days after treatment (DAT). Test initiated September 7, 1993 and concluded January 5, 1994.



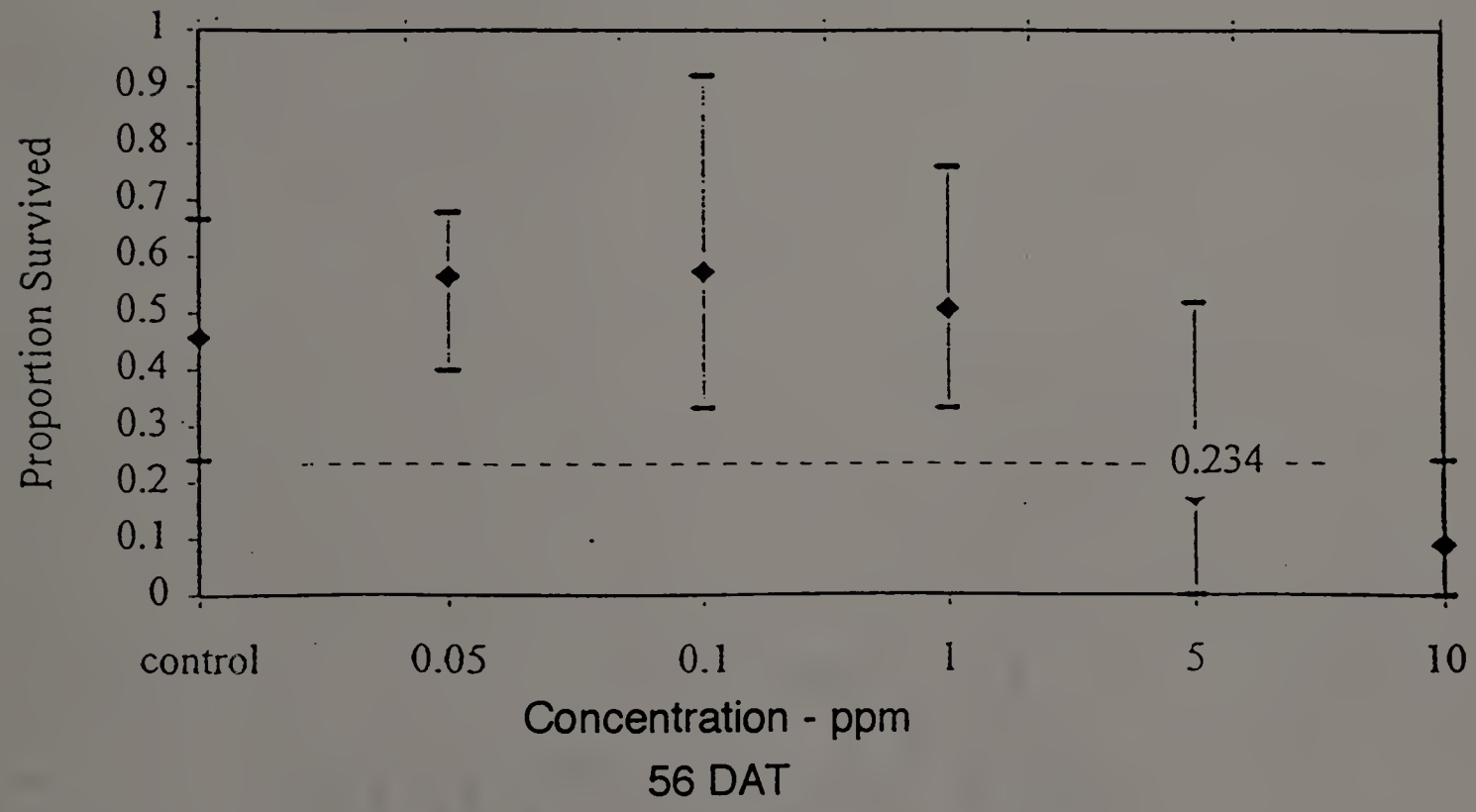


Fig. 24. Dose response plots of the survival of Japanese beetle second instars treated with Margosan-O™ and sampled at 14 and 56 days after treatment (DAT). Test initiated September 7, 1993 and concluded on January 5, 1994. Median survival among treatments (56 and 24%) shown as a dashed line in the center of the plot. Mean survival in treatments shown below this line is significantly different ($p<0.05$) from the mean survival of control groups.

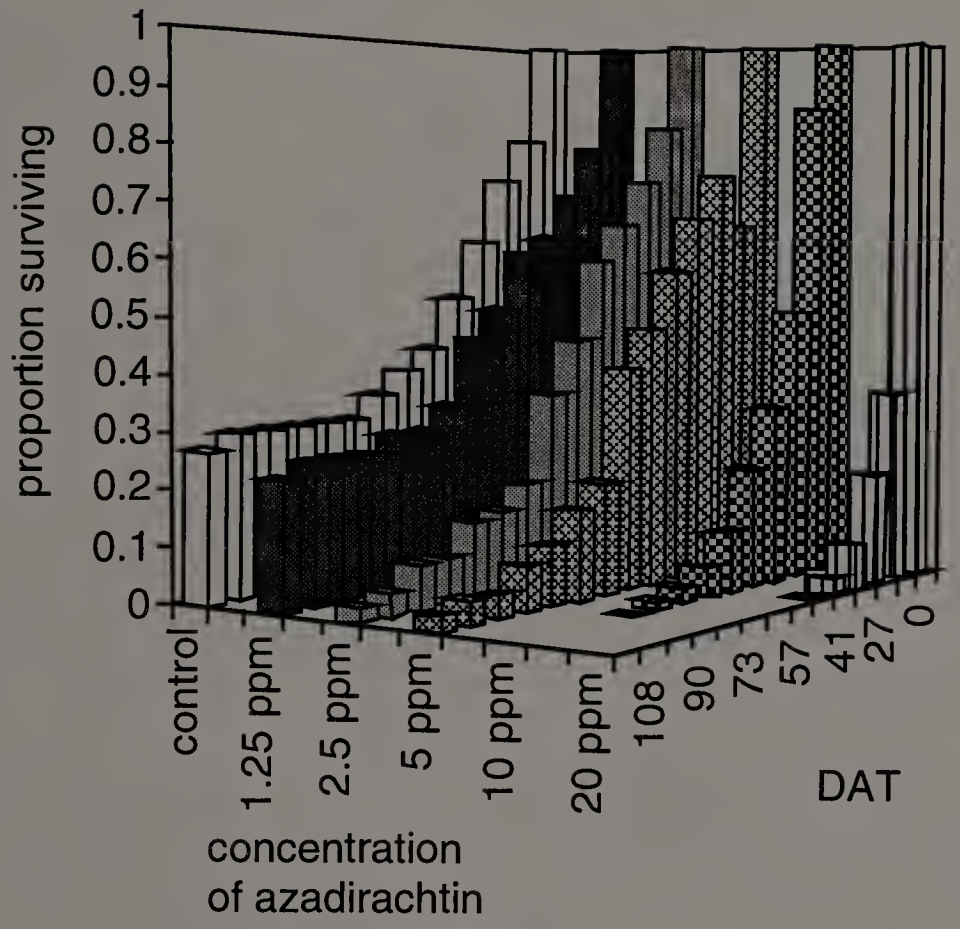


Figure 25. Survival of Japanese beetle third instars treated with Bioneem™ and examined weekly beginning 14 days after treatment (DAT). Third instars from insects reared in the autumn.Test initiated September 29, 1992 and concluded January 8, 1993.

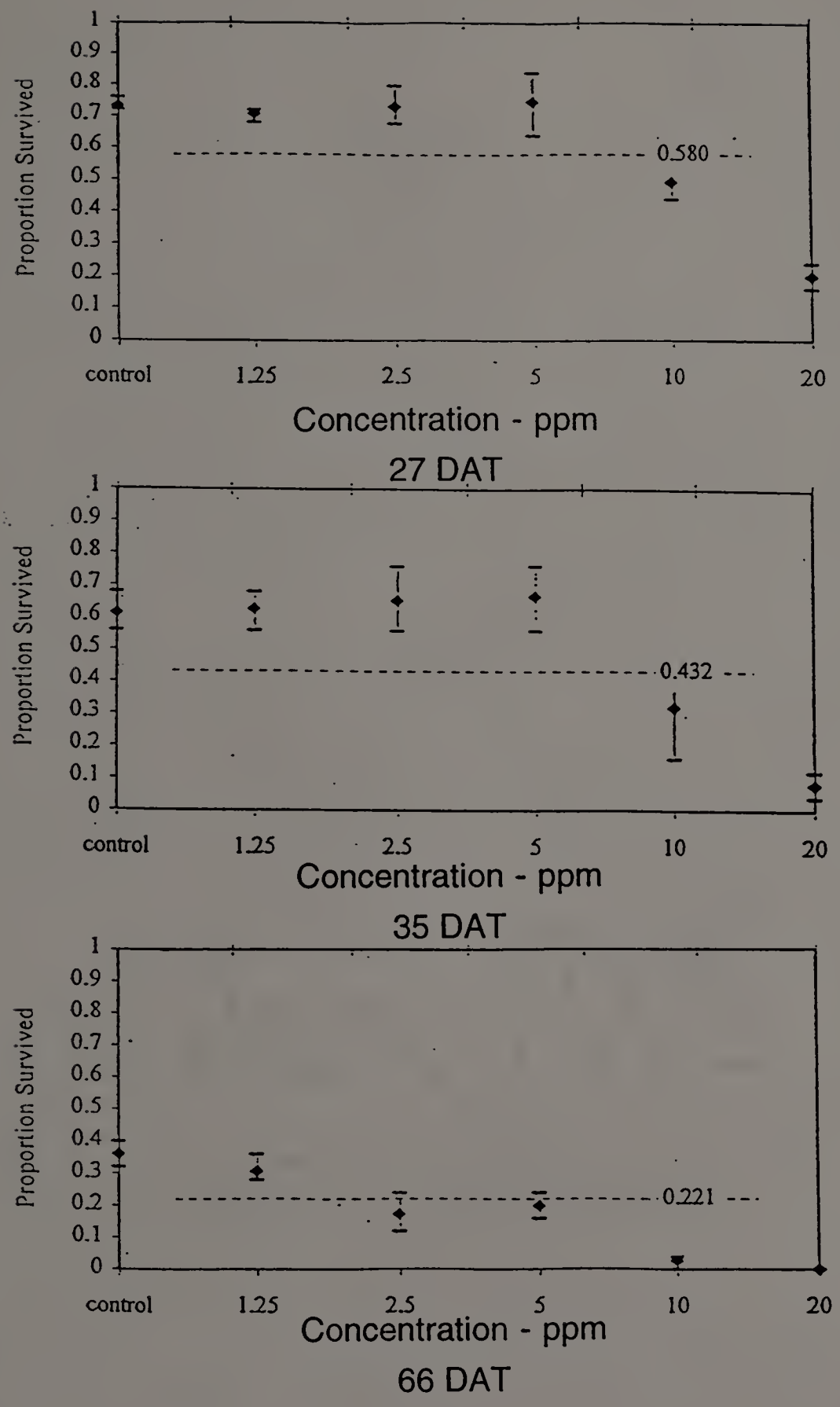


Fig. 26. Dose response plots of the survival of Japanese beetle third instars treated with Bioneem™ and examined for survival at 27, 35 and 66 days after treatment (DAT). Third instars from insects reared in the autumn, 1992.Test initiated September 29, 1992 and concluded January 8, 1993. Median survival among treatments (58, 43 and 22%) shown as a dashed line in the center of the plots. Mean survival in treatments shown below these lines is significantly different ($p<0.05$) from the mean survival of the control groups.

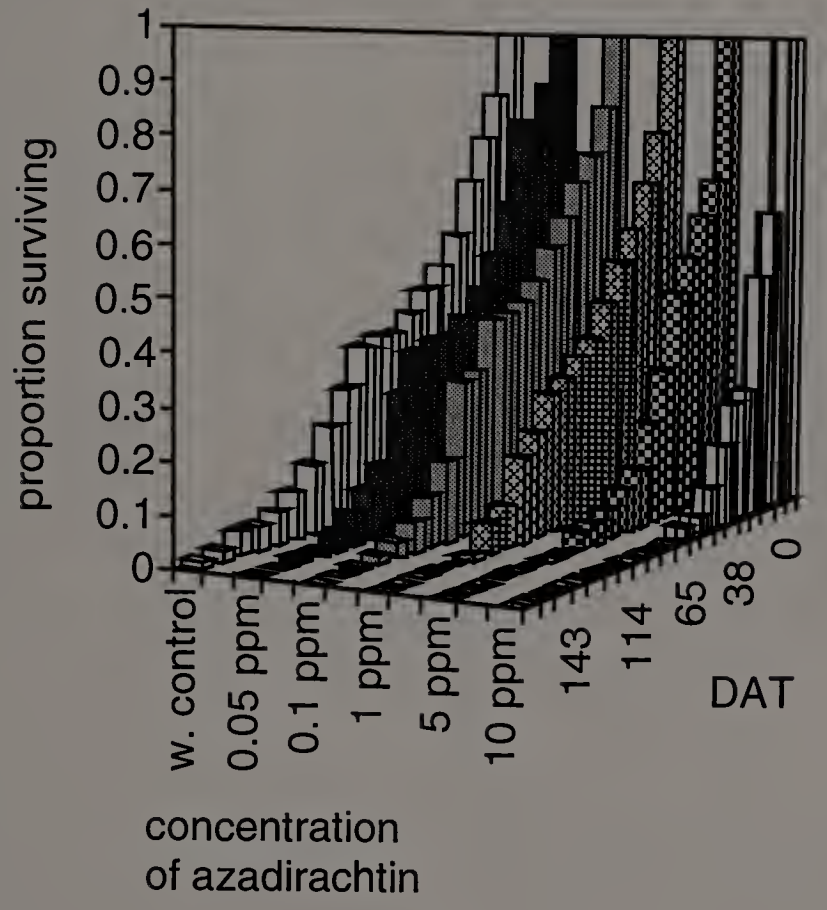


Fig. 27. Survival of field collected Japanese beetle third instars treated with Margosan-O™ and examined weekly beginning 14 days after treatment (DAT). Test initiated September 20, 1993 and concluded January 10, 1994.

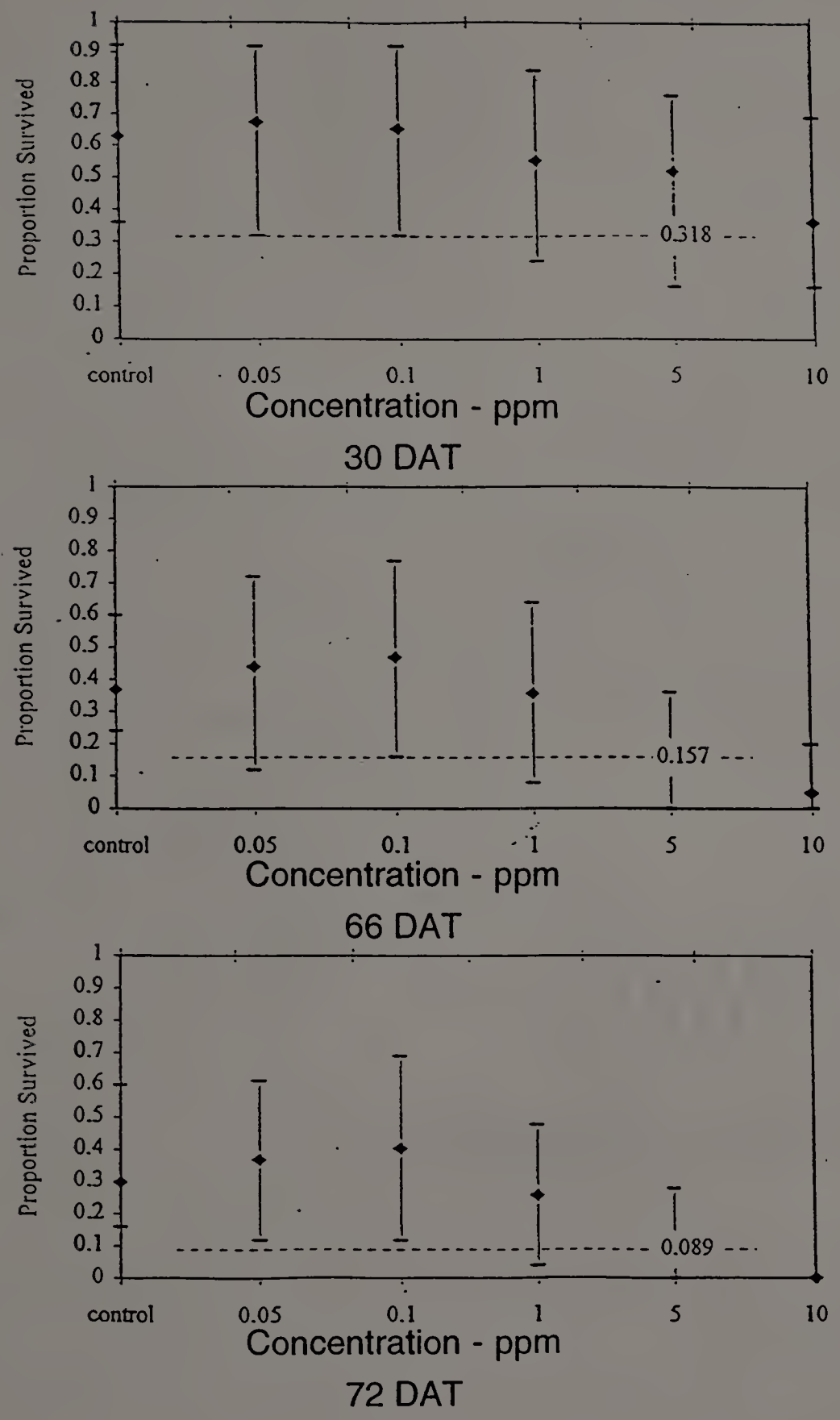


Fig. 28. Dose response plot of the survival of Japanese beetle third instars treated with Margosan-O™ and examined at 30, 66 and 72 days after treatment (DAT). Third instars from insects dug in the autumn, 1993. Test initiated September 20, 1993 and concluded January 10, 1994. Median survival among treatments (32, 16 and 9%) shown as a dashed line near the bottom of each plot. Mean survival in treatments shown below this line is significantly different ($p<0.05$) from the mean survival of the control groups.

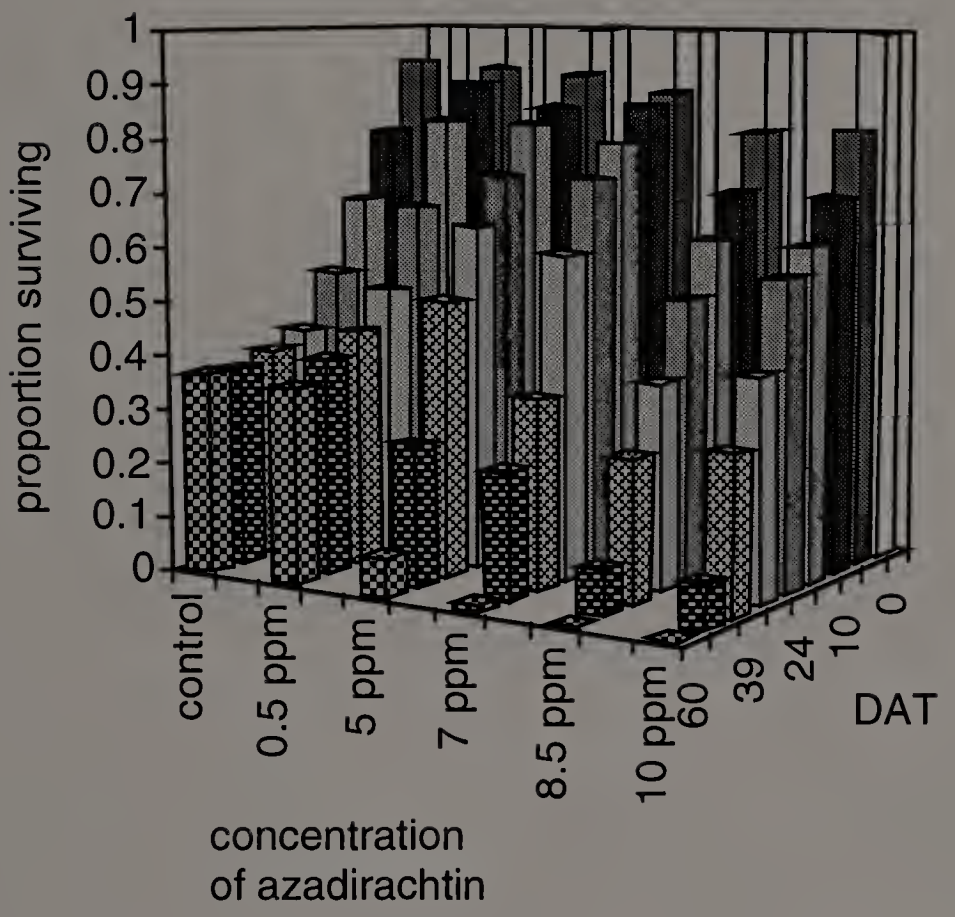


Fig. 29. Survival of field collected Japanese beetle third instars treated with Margosan-O™ and examined weekly beginning at 14 days after treatment (DAT). Test initiated June 4, 1993 and concluded on July 10, 1993.

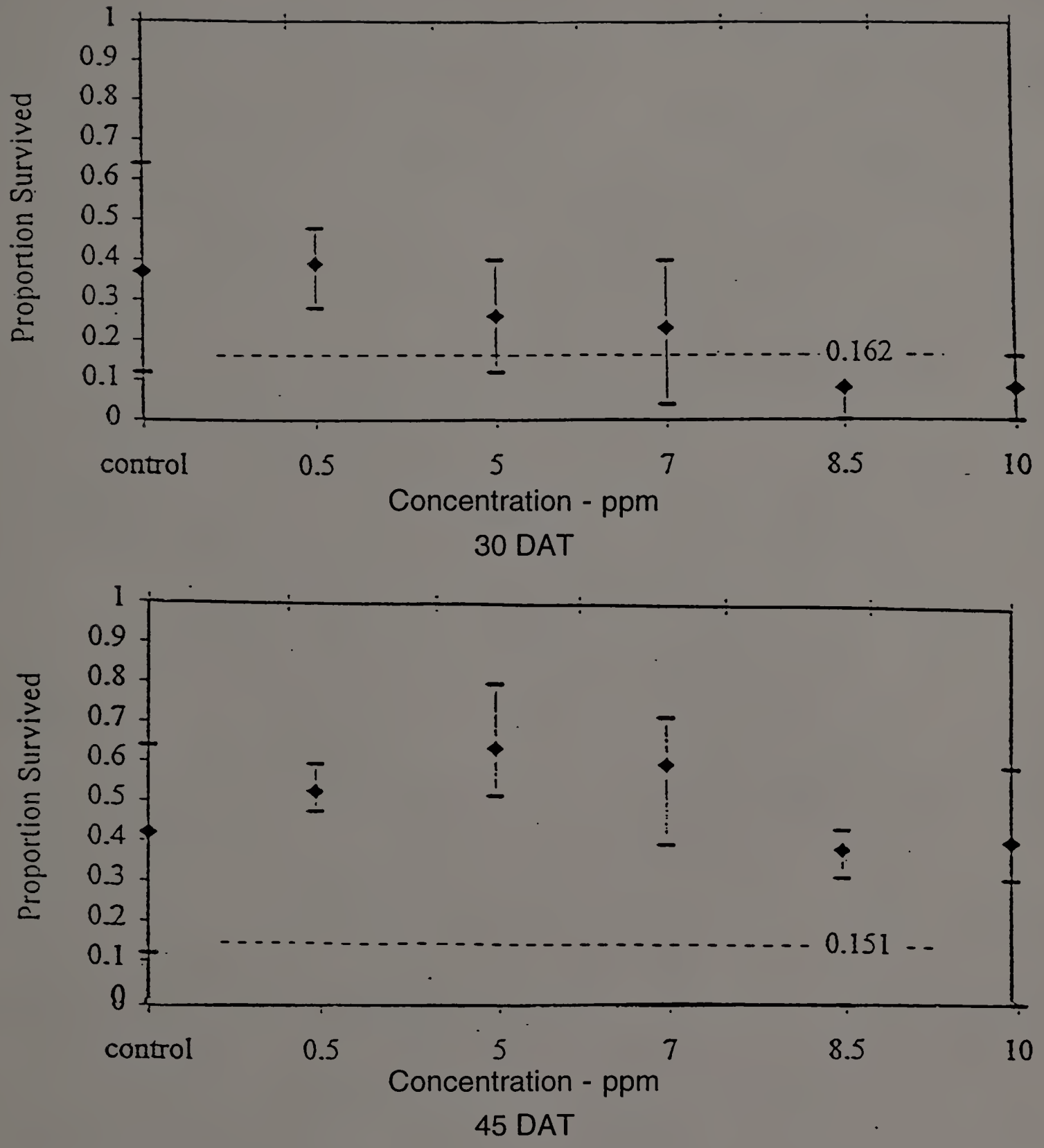


Fig.30. Dose response plot of the survival of field collected Japanese beetle third instars treated with Margosan-O™ and examined for survival at 30 and 45 days after treatment (DAT). Test initiated June 4, 1993 and concluded July 10, 1993.

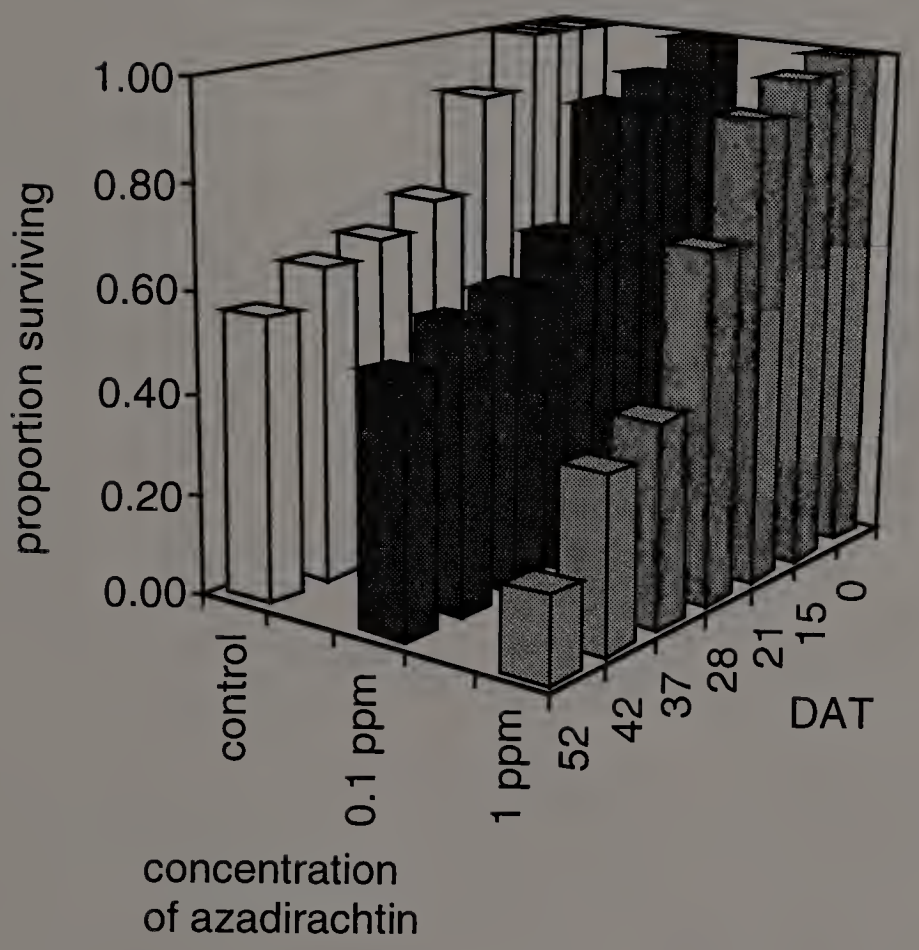


Fig. 31. Survival of field collected Japanese beetle third instars treated with Margosan-O™ and examined weekly beginning 14 days after treatment (DAT). Test initiated April 25, 1994 and concluded June 6, 1994.

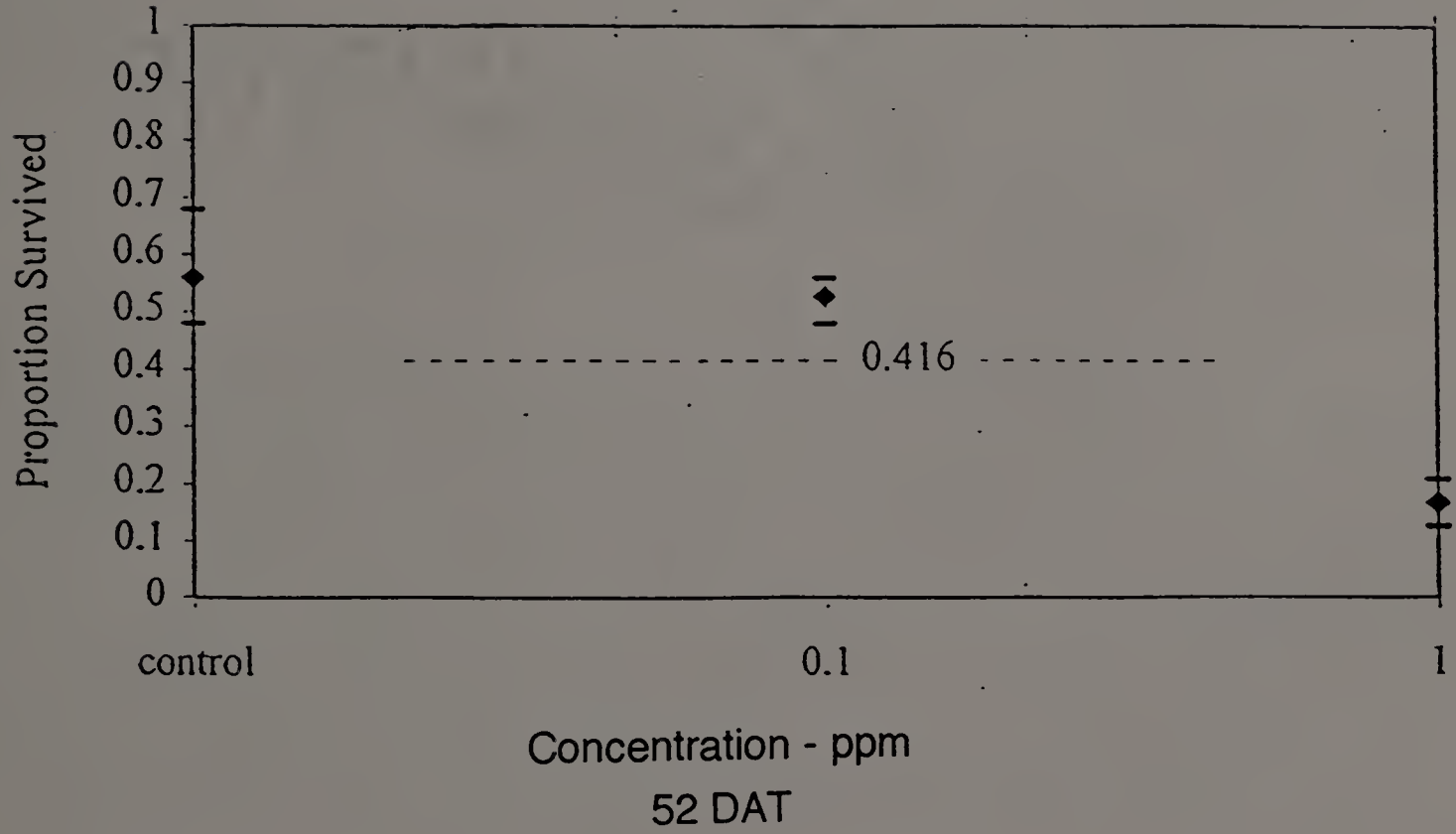


Fig. 32. Dose response plot of the survival of Japanese beetle third instars treated with Margosan-O™ and sampled at 52 days after treatment (DAT). Test initiated April 25, 1994 and concluded June 4, 1994. Median survival among treatments (42%) shown as a dashed line in the center of the plot. Mean survival in treatments shown below this line are significantly different ($p<0.05$) from the mean survival of control groups. Third instars from insects dug in the spring.

CHAPTER IV

FIELD TRIALS OF NEEM FORMULATIONS

Introduction

The commercial formulations of neem extracts tested in the field by our laboratory during 1991 were applied as specified by Agridyne Technologies instructional materials (including commercial labels and supplemental information) during the last two weeks of August, when late-second and early-third instars were feeding actively in turfgrass root systems. Field trials using neem extracts were set out at two sites during August, 1991. In these trials, neem extracts reduced field populations of grubs from 0 to 32% six weeks after treatment, but none of the reductions were statistically different from the control populations (Duncan's MRT, $P<0.05$). Several explanations are possible for these low levels of control: failure of the material to cause mortality, failure of the application method to deliver the formulation to the grubs in the turf, or improper or inappropriate timing of sampling (before lethal effects were evident). To determine whether differences in timing of application or of sampling date would affect treatment results, further trials were conducted in 1993 at Mohawk Meadows Golf Course in Greenfield, MA and in 1994 at the Braintree (MA) Municipal Golf Course.

Materials and Methods

In the initial tests, two trials were conducted in 1991 at the Amherst (MA) Golf Club and on a home lawn in Deerfield, MA. Each trial consisted of 2.6 m x 2.6 m plots replicated 4 times in a complete, randomized block design. Each

block in such a design consisted of one untreated (control) plot and one plot of each material at each treatment level. The mean number of larvae present in the untreated plots was then compared to the mean number of larvae found in each treatment plot, and per cent control was calculated using Abbot's (1925) formula. The turf at each site contained primarily Kentucky bluegrass (*Poa pratensis* L.) although a considerable population of broad-leafed weeds was found at the Deerfield site as well. The application at the Deerfield site was made on 7 August, 1991. The site had received 4 cm of rain 48 h pretreatment and was irrigated with 1.2 cm of water immediately after application. The plot was sampled on 20 September, 6 weeks post treatment. In Amherst, applications were made on 19 August, 1991 with the same plot design. Approximately 2.5 cm of rain fell prior to and during application, and an additional 12 cm of rain fell within 36 h after application. Applications were made with watering cans, using 3 liters of water per plot. Treatments were a neem formulation (Agridyne Technologies, Salt Lake City, UT; numbered compound) at the rate of 20 g, 50 g, and 100 g AI/0.405 ha (active ingredient per 0.405 hectare). Samples in Amherst were taken on 30 September. Two samples were taken in each plot and the results [$(N_1 + N_2/2]$ were combined prior to the calculations of mean counts of insect present in each treatment group. (Two samples per plot are taken in situations when only one sampling visit is anticipated.) Every sample taken was 0.1 m^2 (10 to 15 cm deep) and the number of grubs present in each square was recorded.

The plot selected in 1993 at the Mohawk Meadows Golf Course was designed with larger individual plots to allow for sampling on several dates. Plot size was 2.6 m x 3 m with 5 replications and a complete randomized block design. Turf on the fairway selected for the trial consisted of creeping bentgrass (*Agrostis palustris* Hudson) and annual bluegrass (*Poa annua* L.).

Applications were made on 31 August, 1993 with watering cans, using 3 liters of water per plot, and were watered in by hand (1.2 cm of water) immediately after application. No rain had fallen in the two weeks prior to application. Treatments included Margosan-O 0.3%, at 30 g AI/0.405 ha; Turplex 3%, at 30 g AI/0.405 ha; W. R. Grace 4.5% (a particularly viscous laboratory preparation not available commercially), at 15 g and 30 g AI/0.405 ha; and Bioneem 0.3%, at 15 g and 30 g AI/0.405 ha.The plot was sampled on 14 September, 28 September and 14 October 1993 and again on 2 June 1994. One 0.1 m^2 sample (10 to 15 cm deep) was dug in each plot on each date and the number of grubs present was recorded.

In 1994, a field trial was conducted in Braintree, MA in April to assess the response of spring-treated insects. Plot size was 3 m x 3 m with three replications in a Latin-square design. Insufficient material was available for a larger test. Turf on the fairway selected for the trial consisted primarily of mixed bentgrass species. Applications were made on 19 April with watering cans, using 3 liters of water per plot. All plots were irrigated immediately afterward with 1.2 cm of water. Treatments included Turplex 3%, 30 g AI/0.405 ha and Margosan-O 0.3%, 30g AI/0.405 ha. Rainfall information from the Braintree area was not available for this report. The plot was sampled on 13 June 1994. Two 0.1 m^2 samples 10 to 15 cm deep, were dug from each square in the block design, and the numbers of larvae, pre-pupae and pupae were recorded. The samples were combined as before $[(N_1 + N_2)/2]$ in each plot prior to calculating the mean number of insects (all living insects were included) found in each treatment or control block.

Results of all field trials were analyzed using Super-Anova Software (Abacus Concepts Inc., Berkley, CA) for descriptive statistics and one-way ANOVA (one-tailed test) for differences among treatments. Counts of insects

were entered as compact variables by treatment for analysis. Dunnett's Multiple Comparison (Huck et al. 1974) was selected for comparison of treatments to the control plots. Analysis of field trials during 1991 had been previously analyzed and reported (Vittum et al., 1992) using Duncan's Multiple Range Test (MRT). Outcomes using both tests were the same, and Duncan's MRT is reported for 1991 as a consequence.

Results

The results of the preliminary field trials conducted in 1991 are shown in Table 12. Shown are the mean number ($\pm$ SE) of grubs per 0.1 m^2 samples for each treatment or control (untreated) block. No significant differences among treatments were found for the test in Amherst, and only one, at the 50 g rate, for Deerfield ($P<0.05$). Sample numbers among all treatment and control blocks in the Deerfield plot were greater than the action level of 12 grubs/0.1 m^2. Population reduction never exceeded the 70 - 80% level generally deemed critical for the evaluation of success under field conditions.

The results of field trials conducted at the Mohawk Meadow Golf Course on four sample dates are shown in Table 13. No differences among treatments were reported for this test for any of the sample dates. No significant differences were observed between treatment mean counts of insects at the different sample dates, as was confirmed by analysis using Dunnet's Multiple Comparison Test (Table 13).

The results of the field trial conducted in the spring of 1994 at Braintree Municipal Golf Course are shown in Table 14. Counts of larvae, pre-pupae and pupae present in the samples were pooled for analysis of the results. No significant differences among treatments were found for this trial, as was confirmed by analysis using Dunnet's Multiple Comparison Test (Table 14).

Discussion

The hypothesis that timing of application of neem formulations or of sample dates has a significant effect on grub survival cannot be supported by the results of these field trials. One possible explanation for the failure of the neem products to reduce grub populations is that the material failed to reach its intended target. This could be because the treatment materials were unable to penetrate the thatch layer of turf grasses or because of changes in behavior of the insects which caused them to move out of range of the treatment materials. Villani and Wright (1990) have demonstrated that the behavior of the Japanese beetle varies at different instars, ranging from very little movement in first instars to the considerable mobility of third instars in turf root systems when they are actively feeding in late summer as well as throughout the average 9-month duration of the third instar in the field. These behavioral differences can affect the probability of second and third instars being as high up in the soil profile as first instars when insecticide applications are made.

Low numbers of insects during 1993 made it virtually impossible to draw any conclusions regarding the results of trials at Mohawk Meadow Golf Club. It is not clear that another trial in a year when insect numbers were greater would provide any new information on the efficacy of neem formulations of white grubs. It would appear that either the current formulation or the application technology should be investigated as a possible reason for failure of these products to provide control of Japanese beetle larvae. Perhaps sub-surface placement of the neem formulations would result in increased levels of control beyond those seen in the field trials conducted by our laboratory from 1991 through 1994.

Table 12. Efficacy of a neem formulation (Agridyne Technologies) against Japanese beetle grubs in turf field plots, August, 1991. Treatments (and untreated control) were placed in a randomized complete block design with 4 replications per treatment. Samples taken at 6 weeks after treatment. Results shown as mean number [and standard error (SE)] of insects (grubs) found per 0.1 m^2 sample dug.

Plot	Rate (AI/0.405 ha)[a]	Deerfield[b] mean (± SE) no. live grubs/0.1m^2	Amherst[c] mean (± SE) no. live grubs/0.1m^2
Untreated	0	42.8 ± 6.1 a	11.3 ± 2.8
Neem	20 g	33.0 ± 5.2 ab	16.9 ± 5.3
Neem	50 g	29.0 ± 3.2 b	12.6 ± 4.7
Neem	100 g	31.0 ± 2.8 ab	11.0 ± 3.1

[a]Active ingredient (azadirachtin) per 0.405 hectare

[b]Numbers followed by the same letter are not significantly different from each other. Duncan's Multiple Range Test (P = 0.05)

[c]No significant differences were found among treatments in the Amherst plots

Table 13. Efficacy of different neem formulations against Japanese beetle grubs at Mohawk Meadow Golf Course, 1993-1994. Treatments (and untreated control) were placed in a randomized complete block design with 5 replications on 31 August 1993. Samples taken at 2, 4, 6 and 35 weeks after treatment.

Plot	Rate (AI/0.405 ha)[a]	14 September 1993 mean (±SE) no. live grubs/0.1m^2	28 September 1993 mean (±SE) no. live grubs/0.1m^2
Untreated	0	3.8 ± 1.4 N.S.	3.2 ± 2.7 N.S.
Margosan-O	30 g	2.4 ± 0.8	3.0 ± 1.8
Turplex	30 g	3.0 ± 1.1	3.0 ± 0.8
Grace 4.5%	15 g	3.0 ± 1.5	1.2 ± 0.7
Grace 4.5%	30 g	1.8 ± 0.6	2.2 ± 0.7
Bioneem	15 g	2.6 ± 1.3	0.8 ± 0.6
Bioneem	30 g	1.8 ± 1.6	1.6 ± 0.7

Plot	Rate (AI/0.405 ha)	14 October 1993 mean (±SE) no. live grubs/0.1m^2	2 June 1994 mean (±SE) no. live grubs/0.1m^2
Control	0	2.8 ± 0.6 N.S.	2.8 ± 1.3 N.S.
Margosan-O	30 g	1.5 ± 1.0	2.4 ± 0.5
Turplex	30 g	1.8 ± 1.4	1.4 ± 0.6
Grace 4.5%	15 g	2.5 ± 1.0	4.4 ± 1.8
Grace 4.5%	30 g	1.0 ± 0.6	1.0 ± 0.8
Bioneem	15 g	0	1.6 ± 0.5
Bioneem	30 g	0.75 ± 0.5	1.0 ± 0.6

[a]Active ingredient (azadirachtin) per 0.405 hectare

N.S. - no significant differences (Dunnet's Multiple Comparison, $P< 0.05$)

Table 14. Efficacy of two neem formulations against Japanese beetle grubs in turf field plots, Braintree Golf Course 1994. Treatments (and untreated control) were placed in a latin square (3 replications) on 4 April 1994. Samples taken 13 June, (7 weeks after treatment).

Material	Rate (AI/0.405 ha)[a]	mean (±SE) no. live grubs/0.1m^2
Control	0	15.2 ± 5.7 N.S.
Turplex	30 g	8.8 ± 0.2
Margosan-O	30 g	6.8 ± 1.2

[a]Active ingredient (azadirachtin) per 0.405 hectare

N.S. - no significant differences (Dunnet's Multiple Comparison, $P<0.05$)

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